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(71) Applicant: **Erasmus Universiteit Rotterdam**
3015 GE Rotterdam (NL)

(72) Inventors:
- **Khan, Nisar Ahmed**
  3074 AD Rotterdam (NL)
- **Benner, Robbert**
  2992 SH Barendrecht (NL)

(74) Representative: **Ottevangers, Sietse Ulbe et al**
Vereenigde,
Nieuwe Parklaan 97
2587 BN The Hague (NL)

(54) Fragments of human chorionic gonadotropin (hcg) as immunoregulator

(57) The invention relates to the field of immunology, more specifically to the field of immune-mediated disorders such as allergies, auto-immune disease, transplantation-related disease or inflammatory disease. The invention provides among others an immunoregulator (NMPF), use of an NMPF in preparing a pharmaceutical composition for treating an immune-mediated disorder, a pharmaceutical composition and a method for treating an immune-mediated disorder.

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[0001] The invention relates to the field of immunology, more specifically to the field of immune-mediated disorders such as allergies, auto-immune disease, transplantation-related disease or inflammatory disease.

[0002] The immune system produces cytokines and other humoral factors to protect the host when threatened by inflammatory agents, microbial invasion, or injury. In most cases this complex defence network successfully restores normal homeostasis, but at other times the immunological mediators may actually prove deleterious to the host. Some examples of immune disease and immune system-mediated injury have been extensively investigated including anaphylactic shock, autoimmune disease, and immune complex disorders.

[0003] Recent advances in humoral and cellular immunology, molecular biology and pathology have influenced current thinking about auto-immunity being a component of immune-mediated disease. These advances have increased our understanding of the basic aspects of antibody, B-cell, and T-cell diversity, the generation of innate (effected by monocytes, macrophages, granulocytes, natural killer cells, mast cells, γδ T cells, complement, acute phase proteins, and such) and adaptive (T and B cells and antibodies) or cellular and humoral immune responses and their interdependence, the mechanisms of (self)-tolerance induction and the means by which immunological reactivity develops against auto-antigenic constituents.

[0004] Since 1900, the central dogma of immunology has been that the immune system does not normally react to self. However, it as recently become apparent that auto-immune responses are not as rare as once thought and that not all auto-immune responses are harmful; some responses play a distinct role in mediating the immune response in general. For example, certain forms of auto-immune response such as recognition of cell surface antigens encoded by the major histocompatibility complex (MHC) and of anti-idiotypic responses against self idiotypes are important, indeed essential, for the diversification and normal functioning of the intact immune system.

[0005] Apparently, an intricate system of checks and balances is maintained between various subsets of cells (i.e. T-cells) of the immune system, thereby providing the individual with an immune system capable of coping with foreign invaders. In that sense, auto-immunity plays a regulating role in the immune system.

[0006] However, it is now also recognised that an abnormal auto-immune response is sometimes a primary cause and at other times a secondary contributor to many human and animal diseases. Types of auto-immune disease frequently overlap, and more than one auto-immune disorder tends to occur in the same individual, especially in those with auto-immune endocrinopathies. Auto-immune syndromes may be mediated with lymphoid hyperplasia, malignant lymphocytic or plasma cell proliferation and immunodeficiency disorders such as hypogammaglobulinaemie, selectiv Ig deficiencies and complement component deficiencies.

[0007] Auto-immune diseases, such as systemic lupus erythematosus, diabetes, rheumatoid arthritis, postpartum thyroid dysfunction, auto-immune thromocytopenia, to name a few, are characterised by auto-immune responses, for example directed against widely distributed self-antigenic determinants, or directed against organ- or tissue specific antigens. Such disease may follow abnormal immune responses against only one antigenic target, ore against many self antigens. In many instances, it is not clear whether auto-immune responses are directed against unmodified self-antigens or self-antigens that have been modified (or resemble) any of numerous agents such as viruses, bacterial antigens and haptenic groups.

[0008] There is as yet no established unifying concept to explain the origin and pathogenesis of the various auto-immune disorders. Studies in experimental animals support the notion that auto-immune diseases may result from a wide spectrum of genetic and immunological abnormalities which differ from one individual to another and may express themselves early or late in life depending on the presence or absence of many superimposed exogenous (viruses, bacteria) or endogenous (hormones, cytokines, abnormal genes) accelerating factors.

[0009] It is evident that similar checks and balances that keep primary auto-immune disease at bay are also compromised in immune mediated disorders, such as allergy (asthma), acute inflammatory disease such as sepsis or septic shock, chronic inflammatory disease (i.e rheumatic disease, Sjögrens syndrome, multiple sclerosis), transplantation-related immune responses (graft-versus-host-disease, post-transfusion thrombocytopenia), and many others wherein the responsible antigens (at least initially) may not be self-antigens but wherein the immune response to said antigen is in principle not wanted and detrimental to the individual. Sepsis is a syndrome in which immune mediators, induced by for example microbial invasion, injury or through other factors, induce an acute state of inflammation which leads to abnormal homeostasis, organ damage and eventually to lethal shock. Sepsis refers to a systemic response to serious infection. Patients with sepsis usually manifest fever, tachycardia, tachypnea, leukocytosis, and a localised site of infection. Microbiologic cultures from blood or the infection site are frequently, though not invariably, positive. When this syndrome results in hypotension or multiple organ system failure (MOSF), the condition is called sepsis or septic shock. Initially, micro-organisms proliferate at a nidus of infection. The organisms may invade the bloodstream, resulting in positive blood cultures, or might grow locally and release a variety of substances into the bloodstream. Such substances, when of pathogenic nature

are grouped into two basic categories: endotoxins and exotoxins. Endotoxins typically consist of structural components of the micro-organisms, such as teichoic acid antigens from staphylococci or endotoxins from gram-negative organisms µlike LPS). Exotoxins (e.g., toxic shock syndrome toxin-1, or staphlococcal enterotoxin A, B or C) are synthesised and directly released by the micro-organisms.

[0010] As suggested by their name, both of these types of bacterial toxins have pathogenic effects, stimulating the release of a large number of endogenous host-derived immunological mediators from plasma protein precursors or cells (monocytes/macrophages, endothelial cells, neutrophils, T cells, and others).

[0011] It is in fact generally these immunological mediators which cause the tissue and organ damage associated with sepsis or septic shock. Some of these effects stem from direct mediator-induced injury to organs. However, a portion of shock-associated-organ dysfunction is probably due to mediator-induced abnormalities in vasculature, resulting in abnormalities of systemic and regional blood flow, causing refractory hypotension or MOSF (Bennett et al.).

[0012] The non-obese diabetic (NOD) mouse is a model for auto-immune disease, in this case insulin-dependent diabetes mellitus (IDDM) which main clinical feature is elevated blood glucose levels (hyperglycemia). Said elevated blood glucose level is caused by auto-immune destruction of insulin-producing β cells in the islets of Langerhans of the pancreas (Bach et al. 1991, Atkinson et al. 1994). This is accompanied by a massive cellular infiltration surrounding and penetrating the islets (insulitis) composed of a heterogeneous mixture of CD4+ and CD8+ T lymphocytes, B lymphocytes, macrophages and dendritic cells (O'Reilly et al. 1991).

[0013] The NOD mouse represents a model in which auto-immunity against beta-cells is the primary event in the development of IDDM. Diabetogenesis is mediated through a multifactorial interaction between a unique MHC class II gene and multiple, unlinked, genetic loci, as in the human disease. Moreover, the NOD mouse demonstrates beautifully the critical interaction between heredity and environment, and between primary and secondary auto-immunity, its clinical manifestation is for example depending on various external conditions, most importantly of the micro-organism load of the environment in which the NOD mouse is housed.

[0014] As for auto-immunity demonstrable in NOD mice, most antigen-specific antibodies and T-cell responses are measured after these antigens were detected as self-antigens in diabetic patients. Understanding the role these auto-antigens play in NOD diabetes may further allow to distinguish between pathogenic auto-antigens and auto-immunity that is an epiphenomenon.

[0015] In general, T lymphocytes play a pivotal role in initiating the immune mediated disease process (Sempe et al. 1991, Miyazaki et al. 1985, Harada et al. 1986, Makino et al. 1986). CD4+ T-cells can be separated into at least two major subs ts Th1 and Th2. Activated Th1 cells secr te IFN-γ and TNF-α, whil Th2 cells produce IL-4, IL-5 and IL-10. Th1 cells are critically involved in the generation of effective cellular immunity, whereas Th2 cells are instrumental in the generation of humoral and mucosal immunity and allergy, including the activation of eosinophils and mast cells and the production of IgE (Abbas et al. 1996). A number of studies have now correlated diabetes in mice and human with Th1 phenotype development (Liblau et al. 1995, Katz et al. 1995). On the other hand, Th2 T cells are shown to be relatively innocuous. Some have even speculated that Th2 T cells in fact, may be protective. Katz et al. have shown that the ability of CD4+ T cells to transfer diabetes to naïve recipients resided not with the antigen specificity recognised by the TCR per se, but with the phenotypic nature of the T cell response. Strongly polarised Th1 T cells transferred disease into NOD neonatal mice, while Th2 T cells did not, despite being activated and bearing the same TCR as the diabetogenic Th1 T cell population. Moreover, upon co-transfer, Th2 T cells could not ameliorate the Th1-induced diabetes, even when Th2 cells were co-transferred in 10-fold excess (Pakala et al. 1997).

[0016] The incidence of sepsis or septic shock has been increasing since the 1930's, and all recent evidence suggests that this rise will continue. The reasons for this increasing incidence are many: increased use of invasive devices such as intravascular catheters, widespread use of cytotoxic and immunosuppressive drug therapies for cancer and transplantation, increased longevity of patients with cancer and diabetes who are prone to develop sepsis, and an increase in infections due to antibiotic-resistant organisms. Sepsis or septic shock is the most common cause of death in intensive care units, and it is the thirteenth most common cause of death in the United States. The precise incidence of the disease is not known because it is not reportable; however, a reasonable annual estimate for the United States is 400,000 bouts of sepsis, 200,000 cases of septic shock, and 100,000 deaths from this disease.

[0017] Various micro-organisms, such as Gram-negative and Gram-positive bacteria, as well as fungi, can cause sepsis and septic shock. Certain viruses and rickettsiae probably can produce a similar syndrome. Compared with Gram-positive organisms, Gram-negative bacteria are somewhat more likely to produce sepsis or septic shock. Any site of infection can result in sepsis or septic shock. Frequent causes of sepsis are pyelonephritis, pneumonia, peritonitis, cholangitis, cellulitis, or meningitis. Many of these infections are nosocomial, occurring in patients hospitalised for other medical problems. In patients with normal host defences, a site of infection is identified in most patients. However, in neutropenic patients, a clinical infection site is found in less than half of septic pati nts, probably because small, clinically inapparent infectious in skin or bowel can lead

to bloodstream invasion in the absence of adequate circulating neutrophils. Clearly ther is a need to protect against sepsis or septic shock in patients running such risks.

[0018] Recently, considerable effort has been directed toward identifying septic patients early in their clinical course, when therapies are most likely to be effective. Definitions have incorporated manifestations of the systemic response to infection (fever, tachycardia, tachypn a, and leukocytosis) along with evidence of organ system dysfunction (cardiovascular, respiratory, renal, hepatic, central nervous system, hematologic, or metabolic abnormalities). The most recent definitions use the term systemic inflammatory response syndrome (SIRS) emphasising that sepsis is one example of the body's immunologically-mediated inflammatory responses that can be triggered not only by infections but also by non-infectious disorders, such as trauma and pancreatitis (for interrelationships among systemic inflammatory response (SIRS), sepsis, and infection, see Crit. Care Med. 20:864, 1992; For a review of pathogenic sequences of the events in sepsis or septic shock see N Engl J Med 328:1471., 1993).

[0019] Toxic shock syndrome toxin (TSST-1) represents the most clinically relevant exotoxin, identified as b ing the causative agent in over 90% of toxic shock syndrome cases (where toxic shock is defined as sepsis or septic shock caused by super-antigenic exotoxins). Super antigens differ from "regular" antigens in that they require no cellular processing before being displayed on a MHC molecule. Instead they bind to a semi-conserved region on the exterior of the TCR and cause false "recognition" of self antigens displayed on MHC class II (Perkins et al.; Huber et al. 1993). This results in "false" activation of both the T cell and APC leading to proliferation, activation of effector functions and cytokine secretion. Due to the superantigen's polyclonal activation of T cells, a systemic wide shock results due to excessive inflammatory cytokine release. (Huber et al. 1993, Miethke et al. 1992).

[0020] The inflammatory cytokines involved in sepsis are similar. These immunological mediators are tumor necrosis factor (TNF), interferon gamma (IFN-gamma), nitric oxide (Nox) and interleukin 1(IL-1), which are massively released by monocytes, macrophages and other leukocytes in response to bacterial toxins (Bennett et al., Gutierrez-Ramos et al 1997). The release of TNF and other endogenous mediators may lead to several patho-physiological reactions in sepsis, such as fever, leukopenia, thrombocytopenia, hemodynamic changes, disseminated intravascular coagulation, as well as leukocyte infiltration and inflammation in various organs, all of which may ultimately lead to death. TNF also causes endothelial cells to express adhesion receptors (selectins) and can activate neutrophils to express ligands for these receptors which help neutrophils to adhere with endothelial cell surface for adherence, margination, and migration into tissue inflammatory foci (Bennett et al.). Blocking the adhesion process with monoclonal antibodies prevents tissue injury and improves survival in certain animal models of sepsis or septic shock (Benn tt et al.).

[0021] These findings, both with auto-immune disease, as well as with acute and chronic inflammatory disease, underwrite the postulated existence of cells regulating the balance between activated Th-sub-populations. Possible disturbances in this balance that are induced by altered reactivity of such regulatory T cell populations can cause immune-mediated diseases, which results in absence or over-production of certain critically important cytokines (O'Garra et al. 1997). These Th-sub-populations are potential targets for pharmacological regulation of immune responses.

[0022] In general, immune mediated disorders are difficult to treat. Often, broad-acting medication is applied, such as treatment with corticosteroids or any other broad acting anti-inflammatory agent that in many aspects may be detrimental to a treated individual.

[0023] In general there is a need for better and more specific possibilities to regulate the checks and balances of the immune system and treat immune mediated disorders:

[0024] The invention provides among others an immunoregulator (NMPF) obtainable or derivable from a urinary metabolite of hCG, in particular from nicked forms of b-hCG, or (synthetic) peptide homologues or analogues thereof. These forms of b-hCG have peptide bond cleavages within the b-subunit (Birken et al, Endocrinology 133:1390-1397, 1993). Surprisingly, it has been found that a range of beta-HCG breakdown products provides a cascade of immunoregulators (NPMF) with a host of functions. Even more surprisingly, said immunoregulators are interrelated and derived from one another. The invention provides use of such an NMPF in preparing a pharmaceutical composition for treating an immune-mediated disorder, a phamaceutical composition and a method for treating an immune-mediated disorder. Immune-mediated disorders as described herein include chronic inflammatory disease, such as diabetes type I or II, rheumatic disease, Sjögrens syndrome, multiple sclerosis), transplantation-related immune responses such as graft-versus-host-disease, post-transfusion thrombocytopenia, chronic transplant rejection, pre-eclampsia, atherosclerosis, asthma, allergy and chronic auto-immune disease, and acute inflammatory disease, such as (hyper)acute transplant rejection, septic shock and acute autoimmune disease. Autoimmune diseases are a group of disorders of in general unknown etiology. In most of these diseases production of autoreactive antibodies and/or autoreactive T lymphocytes can be found. An autoimmune response may also occur as manifestation of viral or bacterial infection and may result in severe tissue damage, for example destructive hepatitis because of Hepatitis B virus infection.

[0025] Autoimmune diseases can be classified as or-

gan specific or non-organ specific depending on whether the r sponse is primarily against antigens localised in particular organs, or against wide-spread antigens. The current mainstay of treatment of autoimmune diseases is immune suppression and/or, (because of tissue impairment), substitution of vital components like hormone substitution. However, immunosuppressive agents such as steroids or cytostatic drugs have significant side effects, which limits their application. Now, the use of more specific immunoregulatory drugs is provided by the invention in the treatment of autoimmune disease and other inflammations. Based on the immunoregulatory properties, e.g. the capacities to regulate the Th1/Th2 ratio, to modulate dendritic cell differentiation, their low side-effect profile, and the beneficial clinical effects, etc., it shows these urinary metabolite preparations or synthetic analogues thereof to be very helpful in the treatment of patients with immune-mediated inflammation, such autoimmune disease.

[0026] A non-limiting list of an immune diseases includes: Hashimoto's thyroditis, primary mysxoedema thyrotoxicosis, pernicious anaemia, autoimmune atrophic gastritis, Addison's disease, premature menopause, insulin-dependent diabetes mellitus, stiff-man syndrome, Goodpasture's syndrome, myasthenia gravis, male infertility, pemphigus vulgaris, pemphigoid, sympathetic ophthalmia, phacogenic uveitis, multiple sclerosis, autoimmune haemolytic anaemia, idiopathic thrombocytopenic purpura, idiopathic leucopenia, primary biliary cirrhosis, active chronic hepatitis, cryptogenic cirrhosis, ulcerative colitis, Sjögren's syndrome, rheumatoid arthritis, dermatomyositis, polymyositis, scleroderma, mixed connective tissue disease, discoid lupus erythematosus, and systemic lupus erythematosus.

[0027] In one embodiment, the invention provides an immunoregulator capable of down-regulating Th1 cell levels and/or upregulating Th2 cell levels, or influencing their relative ratio in an animal, said immunoregulator obtainable from urine or other sources of bodily products, such as serum, whey, placental extracts, cells or tissues. Obtainable herein refers to directly or indirectly obtaining said NMPF from said source, NMPF is for example obtained via chemical synthesis or from animal or plant sources in nature.

[0028] In a preferred embodiment, the invention allows regulating relative ratios and /or cytokine activity of lymphocyte subset-populations in a diseased animal (e.g. human), preferably where these lymphocyte subset-populations comprise Th1 or Th2 populations. In general, naive $CD4^+$ helper T lymphocytes (Th) develop into functionally mature effector cells upon stimulation with relevant antigenic peptides presented on the major histocompatibility complex (MHC) class II molecules by antigen-presenting cells (APC). Based on the characteristic set of cytokines produced, Th cells are commonly segregated into at least two different subpopulations: Th1 cells producing exclusively interleukin-2 (IL-2), interferon-gamma (IFN-γ) and lymphotoxin, while Th2 cells produce IL-4, IL-5, IL6, IL10 and IL-13. Thes Th1 and Th2 subsets appear to be extremes in cytokin production profiles and within these polarized subsets, individual Th cells exhibit differential rather than co-ordinated cytokine gene expression. These subsets develop from common Th precursor cells (Thp) after triggering with relevant peptides into Th0 cells producing an array of cytokines, including IL-2, IL-4, IL-5 and IFN-γ. These activated Th0 cells subsequently polarize into the Th1 or Th2 direction based on the cellular and cytokine composition of their microenvironment. Antigen-presenting cells like the various subsets of dendritic cells besides subsets of macrophages largely determine this polarization into Th1 or Th2 subset development. The Th1-TH2 subsets appear to cross-regulate each other's cytokine production profiles, mainly through IFN-γ and IL-10, and from this concept it was rationalized that disturbances in the balance between these two subsets may result in different clinical manifestations [5]. IL-12 is a dominant factor promoting Th1 subset polarization and dendritic cells and macrophages produce IL-12. Moreover, IL-12 induces IFN-γ production by T cells and natural killer (NK) cells. Recently, it was reported that IL-18 acts synergistically with IL-12 to induce Th1 development. Polarization of Th2 cells is critically dependent on the presence of IL-4 produced by T cells or basophils and mast cells. APC-derived IL-6 has also been shown to induce small amounts of IL-4 in developing Th cells. IL-10 and APC-derived prostaglandin E_2 (PGE_2) inhibit IL-12 production and Th1 priming.

[0029] The Th1-Th2 paradigm has been useful in correlating the function of Th1 cells with cell-mediated immunity (inflammatory responses, delayed type hypersensitivity, and cytotoxicity) and Th2 cells with humoral immunity. In general, among infectious diseases, resistance to intracellular bacteria, fungi, and protozoa is linked to mounting a successful Th1 response. Th1 responses can also be linked to pathology, like arthritis, colitis and other inflammatory states. Effective protection against extracellular pathogens, such as helminths, mostly requires a Th2 response, and enhanced humoral immunity may result in successful neutralisation of pathogens by the production of specific antibodies.

[0030] In yet another preferred embodiment, the invention provides an immunoregulator capable of modulating dendritic cell differentiation. The selective outgrowth of Th1 vs. Th2 type cells is dependent on the interaction of precursor Th cells with antigen-presenting cells (APC) carrying the relevant peptide in conjunction with their MHC class II molecules. Cytokines released by the APC and present during the initial interaction between dendritic cells and the pertinent T cell receptor carrying T cells drive the differentiation in to Th1 vs. Th2 subsets. Recently, two different precursors for DC (myeloid vs. lymphoid) have been described in man. Selective development of DC1 from myeloid precursors occurs after stimulation with CD40 Ligand or endotoxin,

and results in high production of IL-12. Lymhoid precursors give rise to DC2 cells aft r CD40Ligand stimulation, and produced IL-1, IL-6 and IL-10. These cytokines are of prim importance in driving the development of the activated Th cell: IL-4 is required for the outgrowth of Th2 type cells which can be greatly enhanced by the presence of IL-10, while selective differentiation to Th1 type cells is exclusively dependent on the presence of IL-12. Since DC1 are characterized by the production of IL-12, they will primarily induce outgrowth of Thl type cells, while DC2 produce IL-10 and selectively promote Th2 development in the presence of exogenous IL-4. It is shown herein that an NMPF as provided by the invention is capable of regulating or modulating DC activity and differentiation, thereby allowing selective differentiation and activity of Th1 and/or Th2 cells.

[0031] In one embodiment, the invention provides an immunoregulator comprising an active component obtainable from a mammalian chorionic gonadotropin preparation said active component capable of stimulating splenocytes obtained from a non-obese diabetes (NOD) mouse, or comprising an active component functionally related to said active compound, for example allowing regulating or modulating DC activity and differentiation, or allowing selective differentiation and activity of Th1 and/or Th2 cells, in case of chronic inflammation, such as diabetes or chronic transplant rejection for example as shown in the detailed description herein wherein said stimulated splenocytes are capable of delaying the onset of diabetes in a NOD-severe-combined-immunodeficient mouse reconstituted with said splenocytes, or wherein said active component is capable of inhibiting gamma-interferon production of splenocytes obtained from a non-obese diabetes (NOD) mouse, or wherein said active component is capable of stimulating interleukine-4 production of splenocytes obtained from a non-obese diabetes (NOD) mouse.

[0032] In another embodiment, the invention provides an immunoregulator comprising an active component obtainable from a mammalian chorionic gonadotropin preparation said active component capable of protecting a mouse against a lipopolysaccharide induced septic shock, for example allowing regulating or modulating DC activity and differentiation, or allowing selective differentiation and activity of Th1 and/or Th2 cells, in case of acute inflammation, such as seen with shock or (hyper)acute transplantation rejection wherein said active component is capable of reducing ASAT or other relevant plasma enzyme levels after or during organ failure. as commonly seen with shock.

[0033] Although said immunoregulator according to the invention is easily obtained as urinary gonadotropin metabolite or break down product from urine, for example wherein said mammalian chorionic gonadotropin preparation is derived from urine, other sources, such as serum, cells or tissues comprising gonadotropin are applicable as well. Also from said sources an immunoregulator according to the invention capable of for example regulating Th1 and/or Th2 cell activity, and/or capable of modulating dendritic cell differentiation, is provided. In particular, as immunor gulator a (synthetic) peptide is provided obtainable of d rivable from beta-HCG, preferably from nicked beta-HCG. Of course, such a peptide, or functional equivalent thereof is obtainable or derivable from other mammalian gonadotropins, as explained herein earlier. Said peptide is for example capable of protecting against septic shock or other immune-mediated disorders. Preferably, said peptide immunoregulator is obtained from a peptide having at least 10 amino acids such as a peptide having an amino acid sequence MTRVLQGVLPALPQVVC or functional fragment (e.g. a breakdown product) or functional analogue thereof. Functional fragments herein relates to the immunoregulatory effect or activity as for example can be measured in the septic shock or NOD mouse experimental model. Fragments can be somewhat (i.e. 1 or 2 amino acids) smaller or larger on one or both sides, while still providing functional activity.

[0034] Functional analogue herein not only relates to analogues or homologues peptides from MIF or MIF-like proteins, from LH or PMSG, or gonadotropin-like proteins, be it modified by glycosylation or modification with unidentified amino acids or non-protein amino acids, but also to synthetic peptide analogues that can be made with peptide synthesis skills known, for example by identification of functional analogues with replacement mapping techniques, PEPSCAN detection technology and so on, and can comprise D- or L-amino acids or modified amino acids at one or more (or all) places in the desired sequence. Also, peptides can be circularised (for example by providing with (terminal) cysteines, dimerised or multimerised, by linkage to lysine or cystein or other side-chains that allow linkaage or multimerisation, repeated, brought in tandem configuration, conjugated or otherwise linked to carriers known in the art, if only by a labile link that allows dissociation.

[0035] Preferably, an immunoregulator as provided by the invention is obtainable or derivable from a gonadotropin from a pregnant mammal, preferably a human, for example obtainable from a pharmacological preparation prepared to contain (placental) gonadotropins such as pregnant mare serum gonadotropin (PMSG) found in serum of pregnant mares, or pregnant mouse uterus extract (PMUE) extracted from uteri of gravid mice or human chorionic gonadotropin (hCG or HCG) found in blood or urine of pregnant women. An NMPF as provided by the invention can be associated with or without gonadotropin as for example present in the urine of first trimester of pregnancy (NMPF) and in commercial hCG preparations (NMPF) has immune regulatory effects.

[0036] In particular, NMPF can inhibit or regulate auto-immune and acute- and chronic-inflammatory diseases. TNF and IFN-gamma are pathologically involved in acute inflammatory disease such as sepsis or septic shock and also in auto-immune and chronic inflamma-

tory diseases. Since NMPF has the ability to regulate T-cell sub-populations and inhibit TNF and IFN-gamma, NMPF can be used to treat, suppress or prevent immune mediator disorders such as sepsis or s ptic shock (acute inflammatory disease) as well as auto-immune disease or chronic inflammatory diseases such as systemic lupus erythematosus, diabetes, rheumatic disease, Sjögrens syndrome, multiple sclerosis, post-partum thyroid dysfunction and thyroid dysfunction related dementia's such as Alzheimer's disease, auto-immune thromocytopenia and others, such as allergies and chronic inflammatory disease and transplantation related immune responses.

[0037] Furthermore, the invention provides detection of genetic predisposition for immune-mediated disorders, whereby individuals with particular isoforms or amino acid variations in HCG or HCG derived peptides or immunoregulators are predisposed for certain disorders. Once known, it is provided by the invention to provide the genetically predisposed individual with the proper peptide immunoregulator via gene therapy

[0038] In particular, an immunoregulator according to the invention is provided wherein said functional fragment comprises a peptide having at least 10 amino acids such as having an amino acid sequence, LQGVLPALPQVVC (β45 + β48), or VLPALPQVVC (β 48) or LQGVLPALPQ (β 45), or a functional analogue thereof, herein also called NMPF-K. Said immunoregulator comprising said peptide (or mixtures of peptides) having the desired length of about at least 10 amino acids (and especially when bound to a larger molecule such as when bound via its cysteine to another beta-HCG fragment) generally regulates Th1/Th2 balance as well as innate immunity during an immune mediated disorder. For example septic shock, LPS induced proliferation of splenocytes or diabetes is accelerated or aggravated. Similar activity is provided by a relative short-chain peptide (third immunoregulator, 3-5 amino acids long) that comprises MTRV or MTR or QVVC or VVC or CLQG or LQGV or LQG (and especially when bound to a larger molecule such as when bound via its cysteine to another beta-HCG fragment).

[0039] More in particular, a first immunoregulator is provided comprising a functional fragment comprising an amino acid sequence VLPALPQVVC or LQGVLPALPQ or functional analogue thereof which counteracts the regulatory activities of another, second immunoregulator according to the invention wherein said functional fragment comprises an amino acid sequence of from 9 to 6 amino acids (herein also called NMPF-Kb), such as VLPALPQ or GVLPALPQ or GVLPALP or VLPALP or functional analogue thereof, which for example is capable of regulating Th1/Th2 balance as well as innate immunity during an immune mediated disorder such that it is capable to reduce the clinical symptoms seen with immune-mediated disorders, such as septic shock, LPS induced proliferation of splenocytes or diabetes, instead of accelerating or aggravating these symptoms of immune-mediated disease, as for example is shown in the detailed description where NMPF-Kb is capable of protecting a mouse against a lipopolysaccharide induced septic shock, or other acute or chronic immune-mediated disorder as explained herein. As there is an overlap between β45 and β48 peptide (β45; LQGVLPALPQ β48: VLPALPQVVC), we also tested denaturated β45+β48 (LQGVLPALPQVVC) peptide for its effect on LPS induced proliferation (in vitro) and anti-shock activity (in vivo) in BALB/c mice. Our results showed that denaturated β45+β48 peptide inhibits LPS induced proliferation and in vivo septic shock. Breakdown products are generated via proteolysis, for example by lysis with leucocyte elastate, and can undergo further notification such as by the activity of (glutathion) transferases. One of the possible breakdown product of β45+β48 peptide is LQG which resembles glutathione (tripeptide of G, C, and Q with L-glutamate having an isopeptide bond with the amino moiety of L-cysteine). We have shown that NMPF also inhibits (toxin) streptozotocin (SZ) induced diabetes in mice through destruction of beta-cells. One of the mechanisms involved in the destruction of pancreatic beta cells is the formation of reactive radicals (ROS, NO etc.) that also play an important role in the pathogenesis of many other diseases like nephropathy, obstructive nephropathy, acute and chronic renal allograft rejection, auto-immune diseases (like SLE, rheumatoid arthritis, diabetes, MS), AIDS, diseases related to angiogenesis, atherosclerosis, thrombosis and type II diabetes mellitus. So, it is likely that NMPF also acts as 'anti-oxidant'. For example breakdown products of β45+β48 such as LQG or CLQG peptides alone or in combination with certain carbohydrates or modified with unidentified amino acids or with nonprotein amino acids such as β-alanine, γ-Aminobutyric acid, Ornithine, etc. posses immunomodulatory activity (NMPF).

[0040] Not wishing to be bound by theory, NMPF-K and NMPF-Kb activity can be described as maintaining a Th1/Th2 balance, whereby NMPF-K acts as if binding to an appropriate receptor but not activating it whereas NMPF-Kb is binding to said receptor and activating it to modulate the Th1/Th2 balance in a beneficial way. NMPF-K and NMPF-Kb are therein both ligands of the same or at least a conformationally similar or alike receptor molecule. Said receptor molecule is now also provided, since it and its acitivity are defined herein by said ligands.

[0041] For example, our results show that NMPF-Kb inhibits sepsis or septic shock caused by endotoxin or by exotoxin. NMPF-Kb as provided by the invention inhibits or counters immune mediated auto-immune diseases, chronic inflammatory diseases as well as acute inflammatory diseases.

[0042] The invention provides a pharmaceutical composition for treating an immune-mediated disorder such as an allergy, auto-immune disease, transplantation-related disease or acute or chronic inflammatory disease and/or provides an immunoregulator (NMPF), for exam-

ple for stimulating or regulating lymphocyte action comprising an active component said active component capable of stimulating splenocytes obtained from a 20-week-old female non-obese diabetes (NOD) mouse, said stimulated splenocytes delaying the onset of diabetes ,in a NOD-severe-combined-immunodeficient (NOD.scid) mouse reconstituted at 8 weeks old with said splenocytes, or comprising an active component functionally related thereto.

[0043] In one embodiment, the invention provides an pharmaceutical composition or immunoregulator wherein said active component is capable of inhibiting gamma-interferon production or stimulating interleukine-4 production of splenocytes obtained from a 20-week-old female non-obese diabetes (NOD) mouse. Clinical grade preparations of gonadotropins such as hCG and PMSG have since long been used to help treat reproductive failure in situations where follicular growth or stimulation of ovulation is desired. Said preparations are generally obtained from serum or urine, and often vary in degree of purification and relative activity, depending on initial concentration in serum or urine and depending on the various methods of preparation used.

[0044] In a particular embodiment, the invention provides a immunoregulator comprising an active component obtainable or derivable from a mammalian CG preparation said active component capable of stimulating splenocytes obtained from a non-obese diabetes (NOD) mouse, or comprising an active component functionally related to said active compound, for example wherein said stimulated splenocytes are capable of delaying the onset of diabetes in a NOD-severe-combined-immunodeficient mouse reconstituted with said splenocytes.

[0045] The invention also provides an immunoregulator wherein said active component is capable of inhibiting gamma-interferon production obtained from a non-obese diabetes (NOD) mouse. The invention also provides an immunoregulator wherein said active component is capable of stimulating interleukine-4 production of splenocytes obtained from a non-obese diabetes (NOD) mouse.

[0046] An immunoregulator as provided by the invention (NMPF) has immune regulatory effects. In particular, NMPF can inhibit or regulate auto-immune and acute- and chronic-inflammatory diseases. TNF and IFN-gamma are pathologically involved in acute inflammatory disease such as sepsis or septic shock and also in auto-immune and chronic inflammatory diseases. Since NMPF has the ability to regulate T-cell sub-populations and inhibit TNF and IFN-gamma, NMPF can be used to treat, suppress or prevent immune mediator disorders such as sepsis or septic shock (acute inflammatory disease) as well as auto-immune disease or chronic inflammatory diseases such as systemic lupus erythematosus, diabetes, rheumatoid arthritis, post-partum thyroid dysfunction, auto-immune thromocytopenia and others, such as allergies and chronic inflammatory disease (i.e. rheumatic disease, Sjögrens syndrome, multiple sclerosis) and transplantation related immune r - spons s. Our results for example show that NMPF-Kb inhibit sepsis or septic shock caused by endotoxin or by exotoxin. NMPF-Kb as provided by the invention inhibits or counters immune mediated auto-immune diseases, chronic inflammatory diseases as well as acute inflammatory diseases.

[0047] The invention thus provides use of an immunoregulator according to the invention for the production of a pharmaceutical composition for the treatment of an immune-mediated-disorder, for example wherein said immune-mediated disorder comprises chronic inflammation, such as diabetes, multiple sclerosis or chronic transplant rejection, wherein said immune-mediated disorder comprises acute inflammation, such as septic or anaphylactic shock or acute or hyper acute transplant rejection, wherein said immune-mediated disorder comprises auto-immune disease, such as systemic lupus erythematosus or rheumatoid arthritis, wherein said immune-mediated disorder comprises allergy, such as asthma or parasitic disease, in particular wherein said immune-mediated disorder comprises an overly strong immune response directed against an infectious agent, such as a virus or bacterium or wherein said immune-mediated disorder comprises pre-eclampsia or another pregnancy related immune-mediated disorder. Use of NMPF-K as contraceptive (e.g. as morning-after-pill or contraceptive vaccine eliciting contraceptive or sterilising antibodies in the vaccinated female mammal) is also provided. Use of NMPF-Kb is provided for facilitating fertility, especially in case where improved implantation is required. Especially, use is provided wherein said treatment comprises regulating innate immunity and/or relative ratios and/or cytokine activity of lymphocyte subset-populations in a treated individual, in particular wherein said subset populations comprise Th1 or Th2 cells. Thus the invention provides a method for treating an immune-mediated-disorder comprising subjecting an animal to treatment with at least one immunoregulator according to the invention, in particular wherein said disorder comprises diabetes or sepsis.

[0048] The invention provides also a method for diagnosing or determining the risk of non-pregnancy related immune disorders associated with Th1/Th2 misbalance as demonstrable by a misbalance between NMPF-K and NMPF-Kb, as for example produced or derived from pituitary derived gonadotropin, especially in age-related disease such as auto-immune and chronic inflammatory disease, such as type II diabetes, rheumatic disease, thyroid dysfunction related mental disease such as dementia's like Alzheimers and others, and atherosclerosis and related disease, said method comprising determining in a sample, preferably a blood or urine sample, the relative ratio of a relative long-chain peptide versus a relative short-chain peptide, said peptides derivable from breakdown of beta-HCG, in particular comprising determining the relative ratio of a relative long-

chain peptide versus a relative short-chain peptid derived from breakdown a peptide having an amino acid sequence MTRVLQGVLPALPQVVC, for example wherein said relative long-chain peptide comprises an amino acid sequence LQGVLPALPQ or GVLPALPQ or VLPALPQ or GVLPALP, in particular wherein said relative short-chain peptide comprises MTRV or MTR or QVVC or VVC or LQGV or LQG. Detection of said long-chain peptides and short chain peptides, be it modified by glycosylation or modification with unidentified amino acids or non-protein amino acids is preferably achieved by immunological detection methods as known in the art.

[0049] The invention provides also a method for diagnosing or determining the risk of a pregnancy related immune-mediated disorder such as pre-eclampsia, and the outcome of pregnancy and/or pregnancy related immune disease (such as gestation diabetes mellitus (GDM)) comprising determining in a sample, preferably a urine sample, the relative ratio of a relative long-chain peptide versus a relative short-chain peptide, said peptides derivable from breakdown of beta-HCG, in particular comprising determining the relative ratio of a relative long-chain peptide versus a relative short-chain peptide derived from breakdown a peptide having an amino acid sequence MTRVLQGVLPALPQVVC, for example wherein said relative long-chain peptide comprises an amino acid sequence LQGVLPALPQ or GVLPALPQ or VLPALPQ or GVLPALP, in particular wherein said relative short-chain peptide comprises MTRV or MTR or QVVC or VVC, or LQGV or LQG.

[0050] Anecdotal observations and laboratory studies indicated previously that hCG might have an anti-Kaposi's sarcoma and anti-human-immunodeficiency-virus effect (Treatment Issues, July/August 1995, page 15. It has been observed that hCG preparations have a direct apoptotic (cytotoxic) effect on Kaposi's sarcoma (KS) in vitro and in immunodeficient patients and mice and a prohematopoetic effect on immunodeficient patients (Lunardi-Iskandar et al., Nature 375, 64-68; Gill et al., New. Eng. J. Med. 335, 1261-1269, 1996; US patent 5677275), and a direct inhibitory antiviral effect on human and simian immunodeficiency virus (HIV and SIV) (Lunardi-Iskandar et al., Nature Med. 4, 428-434, 1998, US patent 5700781). Said cytotoxic and anti-viral effects have also been attributed to an unknown hCG mediated factor (HAF), present in clinical grade preparations of hCG. However, commercial hCG preparations (such as CG-10, Steris Profasi, Pregnyl, Choragon, Serono Profasi, APL), have various effects. Analysis of several of these, (AIDS, 11: 1333-1340, 1997) for example shows that only some (such as CG-10, Steris Profasi) are KS-killing whereas others (Pregnyl, Choragon, Serono Profasi) were not. Secondly, recombinant subunits of (á or β) hCG were killing but intact recombinant hCH not. It was also found that the killing effect was also seen with lymphocytes. Therapy of KS has recently been directed at using beta-hCG for its anti-tumour effect Eur. J. Med Res. 21: 155-158, 1997, and it was reported that-the beta-core fragment isolat d from urine had th highest apoptotic activity on KS cells (AIDS, 11: ,713-721, 1997). Recently, Gallo et. al. reported anti-Kaposi's Sarcoma, anti-HIV, anti-SIV and distinct hematopoietic effects of clinical grade crude preparations of human chorionic gonadotropin (hCG) (Lunardi-Iskandar et al. 1995, Gill et al. 1996, Lunardi-Iskandar et al. 1998). In contrast to their previous studies, it is also claimed that the anti-tumour and anti-viral activity of hCG preparation is not due to the native hCG heterodimer, including its purified subunits or its major degradation product, the β-core; instead the active moiety resides in an as yet unidentified hCG mediated factor (HAF). Whatever the true factor may be, these unidentified factors in several hCG preparations have anti-tumour activity through the selective induction of apoptosis, besides direct cytotoxic effects on the tumour cells. Furthermore, they postulated that the anti-tumour activity could not be due to an immune-mediated response, since there was no infiltration of the tumour with mononuclear cells.

[0051] Moreover, the reported pro-hematopoietic effect of clinical grade hCG was noted in clinical studies in humans infected with HIV, (Lunardi-Iskandar et al. 1998) indicating that the hematopoietic effect is indirect, and caused by rescuing CD4+ cells otherwise killed by HIV through the anti-HIV activity of hCG.

[0052] The invention provides an immunoregulator or a pharmaceutical composition for treating an immune-mediated disorder obtainable from a hCG preparation or a fraction derived thereof. The effects of said immunoregulator include a stimulating effect on lymphocyte populations (such as found in peripheral lymphocytes, thymocytes or splenocytes), instead of cytotoxic or anti-viral effects. The invention provides a method for treating an immune-mediated-disorder comprising subjecting an animal to treatment with at least one immunoregulator obtainable from a pregnant mammal. Said treatment can be direct, for example treatment can comprise providing said individual with a pharmaceutical composition, such as a hCG or PMSG preparation, comprising an immunoregulator as provided by the invention. It is also possible to provide said pharmaceutical composition with a fraction or fractions derived from a pregnant animal by for example sampling urine or serum or placental (be it of maternal or foetal origin) or other tissue or cells and preparing said immunoregulator comprising said active component from said urine or serum or tissue or cells by fractionation techniques known in the art (for example by gel permeation chromatograpy) and testing for its active component by stimulating a NOD mouse or its splenocytes as described. In particular, said preparation or component is prefarably derived from a pregnant animal since an embryo has to survive a potentially fatal immunological conflict with its mother: developing as an essentially foreign tissue within the womb without triggering a hostile immune attack. So, to prevent this rejection "allograft" the immunological interaction be-

tween mother and fetus has to be suppress d, either for instance through lack of fetal-antig n presentation to maternal lymphocytes, or through functional "suppression" of the maternal lymphocytes. If fetal antigens are presented, mat rnal immun responses would be biased to the less damaging, antibody-mediated T helper 2 (Th2)-type. This would suggest that pregnant women are susceptible to overwhelming infection, which is not the case. Female individuals during pregnancy maintain or even increase their resistance to infection. Moreover, while said individuals normally are more susceptible to immune diseases than male individuals, especially autoimmune diseases, during pregnancy they are more resistant to these diseases.

[0053] The invention also provides a method for in vitro stimulation of lymphocytes and transferring said stimulated lymphocytes as a pharmaceutical composition to an animal for treating said animal for an immune mediated disorder. In a particular embodiment of the invention a pharmaceutical composition is provided comprising lymphocytes stimulated in vitro with an immunoregulator provided by the invention.

[0054] In a preferred embodiment of the invention, said disorder comprises diabetes, yet other immune mediated disorders, such as acute and chronic inflammation, can also be treated. In yet another preferred embodiment, said disorder comprises sepsis or septic shock. The invention provides a method of treatment for an animal, preferably wherein said animal is human.

[0055] In a particular embodiment, a method provided by the invention is further comprising regulating relative ratios and /or cytokine activity or cytokine expression or marker expression of lymphocyte subset-populations in said animal, such as subset-populations that comprise Th1 or Th2 cells, or Th3 or Th8 cells, or other effector or regulatory T-cell populations. The invention also provides an immunoregulator for use in a method according to the invention, and use of said immunoregulator, preferably obtainable from a pregnant mammal, for the production of a pharmaceutical composition for the treatment of an immune-mediated-disorder, preferably selected from a group consisting of allergies, auto-immune disease (such as systemic lupus erythematosus or rheumatoid arthritis), transplantation-related disease and acute (such as septic or anaphylactic shock or acute or hyper acute transplant rejection) and chronic inflammatory disease (such as atherosclerose, diabetes, multiple sclerosis or chronic transplant rejection). Furthermore, the invention provides a use according to the invention wherein said immune-mediated disorder comprises allergy, such as asthma or parasitic disease, or use according to the invention wherein said immune-mediated disorder comprises an overly strong immune response directed against an infectious agent, such as a virus or bacterium. Often in most of these diseases production of autoreactive antibodies and/or autoreactive T lymphocytes can be found mounting or being part of a too strong immune response. This is for example seen with parasitic dis ase, wh re IgE production is overly strong or which dis ase is Th2 dependent, and detrimental for the organism, but also with (myco)bacterial inf ctions such as TBC or leprosy. An autoimmune response may also occur as manifestation of viral or bacterial infection and may result in severe tissue damage, for example destructive hepatitis because of Hepatitis B virus infection, or as seen with lymphocytic choriomeningitis virus (LCMV) infections. Said overly strong immune response is kept at bay with an immunoregulator as provided by the invention. Yet other use as provided by the invention relates to treatment of vascular disease, whereby radical damage (damage caused by radicals) to cells and tissue is prevented or repaired by treatment with NMPF according to the invention; whereby NMPF also acts as anti-oxidant directly or indirectly. For example, a determining event in the pathogenesis of diabetes I is the destruction of insulin-producing pancreatic beta cells. There is strong evidence that the progressive reduction of the beta-cell mass is the result of a chronic autoimmune reaction. During this process, islet-infiltrating immune cells, islet capillary endothelial cells and the beta cell itself are able to release cytotoxic mediators. Cytokines, and in particular nitric oxide (NO), are potent beta-cell toxic effector molecules. The reactive radical NO mediates its deleterious effect mainly through the induction of widespread DNA strand breaks, other radicals, such as oxygen, through their effects on lymfocyte sub-populations such as Th1 and Th2 cells. This initial damage triggers a chain of events terminating in the death of the beta cell and disarray of the immune response.

[0056] Furthermore, an immunoregulator according to the invention is capable of regulating radical induced or directed cell-cell interactions or cell responses, specifically those interactions or responses of an immunological nature, e.g. related to regulating interactions of the innate or adaptive immune system. Not wishing to be bound by theory, there are two arms of the immune system: the innate (non-specific) and adaptive (specific) systems, both of which have cellular and humoral components. Examples of cellular components of the innate immune system are monocytes, macrophages, granulocytes, NK cells, mast cells, gd T cell etc, while, examples of humoral components are lysozyme, complement, acute phase proteins and mannose-binding lectin (MBL). The major cellular components of the adaptive immune system are T and B cells, while examples of humoral components are antibodies. The adaptive system has been studied most because of its specificity, effectiveness at eliminating infection and exclusive presence in higher multicellular organisms. The innate system is often considered primitive and thought to be 'unsophisticated'. However, the innate system not only persists but could also play a critical role in one of the most fundamental immune challenges - viviparity. The innate system instigates an immune response by processing and presenting antigen in association with

major histocompatibility complex (MHC) class I and II molecules to lymphocytes. Full response often requires adjuvant (such as ndotoxin), which, through interaction with the innat immune system, produce costimulatory surface molecules or cytokines. This determines the biological significance of antigens and communicates this information to the adaptive system. So it instructs the adaptive system to either respond or not. So these two great arms of immune system not only influence each other but also regulate each other at least at the cellular level through for example cytokines and co-stimulatory molecules etc.

[0057] There are many physiological conditions and immune pathologies where these two systems are involved separately or in combination. For example, it has been shown that in pregnancy the maternal innate immune system is more stimulated, or for it has been proposed that type II diabetes mellitus is a disease of a chronic hyperactive innate immune system. Another example is the involvement of the innate immune system in listeriosis. Dysregulation in the adaptive immune system may also lead to immune diseases like systemic or organ-specific autoimmunity, allergy, asthma etc, but it can also play a role in the maintenance of pregnancy and in the prevention of "allograft" rejection.

[0058] As mentioned above, the adaptive system has been studied most because of its specificity, effectiveness at eliminating infection, and exclusive presence in higher multicellular organisms. Its regulation has also been studied most. For example, it well known that the cytokine micro-environment plays a key role in T helper cell differentiation toward the Th1 or Th2 cell type during immune responses. IL-12 induces Th1 differentiation, whereas IL-4 drives Th2 differentiation. Recently it has also been shown that subsets of dendritic cells (DC1, DC2) provide different cytokine microenvironments that determine the differentiation of either Th1 or Th2 cells. In addition, negative feedback loops from mature T helper cell responses also regulate the survival of the appropriate dendritic cell subset and thereby selectively inhibit prolonged Th1 or Th2 responses. Moreover, development of Th1 responses can be antagonized directly by IL-4 and indirectly by IL-10, which inhibits the production of IL-12 and interferon-g-inducing factor (IGIF) by macrophages stimulated by the innate immune response. Th2 cells dependent on IL-4 to proliferate and differentiate have been implicated in allergic and atopic manifestations, and in addition through their production of IL-4 and IL-10, have been suggested to play a role in tolerance. Specifically, it has been suggested that Th1 to Th2 switch may prevent the development of organ-specific autoimmune pathologies and required for the maintance of pregnancy. Recently it has become clear that distinct subsets of regulatory T cells are responsible for regulating both Th1 and Th2 responses and prev nt the development of immune pathologies. One of the common features of many of these regulatory T cells is that their function is at least in part due the action of TGF-beta; this would be in keeping with the ability of TGF-beta to inhibit both Th1 and Th2 development while IL-10 could preferentially inhibit Th1 alone.

[0059] The selective outgrowth of Th1 vs. Th2 type cells is dependent on the interaction of precursor Th cells with antigen-presenting cells (APC) carrying the relevant peptide in conjunction with their MHC class II molecules. Cytokines released by the APC and present during the initial interaction between dendritic cells and the pertinent T cell receptor carrying T cells drive the differentiation in to Th1 vs. Th2 subsets. Recently, two different precursors for DC (myeloid vs. lymphoid) have been described in man. Selective development of DC1 from myeloid precursors occurs after stimulation with CD40Ligand or endotoxin, and results in high production of IL-12. Lymhoid precursors give rise to DC2 cells after CD40Ligand stimulation, and produced IL-1, IL-6 and IL-10. These cytokines are of prime importance in driving the development of the activated Th cell: IL-4 is required for the outgrowth of Th2 type cells which can be greatly enhanced by the presence of IL-10, while selective differentiation to Th1 type cells is exclusively dependent on the presence of IL-12. Since DC1 are characterized by the production of IL-12, they will primarily induce outgrowth of Th1 type cells, while DC2 produce IL-10 and selectively promote Th2 development in the presence of exogenous IL-4.

[0060] NMPF as provided by the invention is able to regulate the Th1/Th2 balance in vivo (BALB/c, NOD) and in vitro. In dominant Th1 phenotype models like NOD, NMPF (like NMPF-P and its fractions) amongst others down-regulates the IFN-gamma production (in vivo/in vitro) and promote the IL-10 and TGF-beta production, in contrast to IL-4 production, which indicates the induction of regulatory cells like Th3 and Tr1 by NMPF. These regulatory cells may play role in the therapeutic effects of NMPF in immune and inflammatory diseases and immune tolerance. Furthermore, the invention provides an immunoregulator selected by a method according to the invention, a pharmaceutical composition comprising such a selected immunoregulator, and the use of said for the preparation of a pharmaceutical composition for the treatment of an immune-mediated disorder.

[0061] Purified NMPF is used to produce monoclonal antibodies and/or other specific reagents thereby facilitating the design of an NMPF-specific quantitative immuno-assay. Also single chain F_v fragments are isolated by using the phage display technology with the use of a phage library containing a repertoire comprising a vast number of different specificities.

[0062] The invention is further explained in the detailed description without limiting the invention thereto.

Detailed description

Immunoregulator (NMPF)

Introduction

[0063] The immune system has two arms: the non-specific (innate) and specific (adaptive) immune defense, both of which have cellular and humoral components. T and B cells account for the antigen-specific cellular and humoral (antibodies) immune defense. On the other hand, monocytes/macrophages, granulocytes, NK cells, mast cells and likely also gd T cells are the cellular components of the innate immune system, while complement, acute phase proteins, lysozyme and mannose-binding lectin (MBL) are major humoral components of the innate immune system. The adaptive system has been studied most because of its specificity and lasting effectiveness in eliminating infections. The innate system is thought to play a critical role in the most fundamental immune challenge in mammals: viviparity. The innate system instigates an immune response by processing and presenting antigen in association with major histocompatibility complex (MHC) class I and II molecules to lymphocytes, the so called signal 1. Full responses often require adjuvants (such as endotoxin), which, through interaction with the innate immune system, produce signal 2, in the form of costimulatory surface molecules or cytokines. Signal 2 appears to determine the biological significance of antigens and communicates this information to the adaptive system. In fact, it is believed that this signal 2 instructs the adaptive system to either respond or not (Immunology Today 20, 114-118). So, the innate system is an integral part of the specific immune defense.

During pregnancy there are increased numbers of monocytes and granulocytes from the first trimester onwards. It has been found that, in normal pregnancy, circulating monocytes and granulocytes have activated phenotypes, in some ways comparable with changes observed in systemic sepsis (Am. J. Obstet. Gynecol. 179, 80-86). Others have shown increased monocyte phagocytosis and respiratory burst activity. Monocyte surface expression of the endotoxin receptor CD14 is increased, and in response to endotoxin monocytes from normal pregnant women produce more of the proinflammatory type I cytokine IL-12 (Immunology Today 20, 114-118). Other studies have similarly found granulocyte activation in pregnancy as well as changes in plasma levels of soluble innate factors typical of an acute phase response (Am. J. Reprod. Immunol. Microbiol. 15, 19-23).

[0064] During pregnancy the maternal immune system is modulated, resulting in suppression of maternal immune responses against the fetus, while maintaining the mother's resistance to infection. We have shown the presence of immunoregulator (IR, WO99-59617) which we named in this document NMPF (Natural immunoModulatory Pregnancy-Factor(s)) that regulate both innate and adaptive immune systems in a stimulatory and antagonistic way (WO99-59617). These factors include, but are not limited to, commercial hCG preparations derived from human pregnancy urine, b-hCG preparations, certain peptides of b-hCG, certain combinations of b-hCG peptides and certain gel filtration chromatography fractions of commercial hCG preparations and human pregnancy urine. Balance in these factors is crucial for proper regulation of the maternal immune system. For example, the over-activation of the innate system can cause problems in the progression of the pregnancy itself. Pre-eclampsia is one of such condition characterized by hyperactivation of the innate immune system. Recently it has been also suggested that the chronic misbalance between the two immune systems could be the basis of type II diabetes (non-insulin dependent diabetes mellitus) and other diseases as well (WO99-59617).

Several cytokines have been proposed to play an important role in balancing the immune system. One such cytokine which plays an important role in the innate immune defense and in the regulation of inflammatory responses is macrophage migration inhibitory factor (MIF).

MIF was originally identified by its ability to prevent the migration of macrophages out of capillary tubes. Since then, the expression of MIF activity has been found at a variety of inflammatory loci, suggesting its role in regulating the function of macrophages in host defense (Science 153, 80-82; J. Exp. Med. 137, 275-288). First described as a T-cell cytokine, recently, MIF is identified to be a peptide also released by pituitary cells in response to infection and stress (Nature 365, 756-759; Nature 377, 68-71). Originally considered to be the target of MIF action, monocytes and macrophages have been found to be a main source of MIF that is released after exposure to bacterial endo- and exotoxins and to cytokines. Once released, MIF induces the expression of proinflammatory mediators by macrophages and activated T cells, thereby strongly promoting inflammatory and immune responses (Nature Medicine 6,164-170). The critical regulatory role of MIF within the immune system is further underscored by the finding that MIF is induced by glucocorticoids and has the unique ability to override the anti-inflammatory and immunosuppressive effects of glucocorticoids on macrophages and T cells. Thus, MIF and glucocorticoids function as a physiological counter-regulatory dyad that controls host inflammatory and immune responses (Proc. Natl. Acad. Sci. USA 93, 7849-7854). Anti-MIF antibodies reduce the inflammation in experimental models of glomerulonephritis, arthritis, and allograft rejection, confirming the role of MIF in the regulation of inflammatory responses. Elevated concentrations of MIF have also been detected in alveolar air spaces of patients with the adult respiratory distress syndrom (ARDS). Recent studies hav also shown that MIF is an important m diator of lethal en-

dotoxemia and staphylococcal toxic shock, playing a critical role in the pathogenesis of septic shock. Besides the functions in the immune system, MIF has also other activities. For instance, MIF mRNA and protein are expressed in brain, embryonic eye lens and differentiating epidermal cells, suggesting its pivotal role in the regulation of the neuroendocrine system, cell growth and differentiation. A number of reports showed the presence of MIF in various organs and tissues: dermal vessels constitutively express MIF and can be strongly activated to express MIF in acute/chronic inflammations such as eczema and psoriasis. MIF expression on endothelium may provide an important differentiogenic signal for mononuclear phagocytes on their way to the tissue site. One of the mechanisms of immune regulation that we detect during pregnancy is through modulation of the innate and adaptive immune defenses by NMPF. By way of example, but not limited to, acting directly or indirectly on regulatory cells of the APC compartment (such as DC1, DC2) or on lymphocytes (regulatory T cells), NMPF biases activated T lymphocytes towards Th2 immune response. The suppression of Th1 immune responses may be compensated by the stimulation of the innate immune defense by NMPF which could explain the maintenance of maternal resistance to infection. Recently, it has been shown that in some instances such compensatory mechanism (stimulation of innate immunity) could be more dominant and may account for abnormal pregnancy: pre-eclampsia. Pre-eclampsia is a common, pregnancy-specific syndrome defined by clinical findings of elevated blood pressure combined with proteinuria and edema. The incidence has been reported to be between two and seven per cent of all pregnancies. The clinical findings become manifested mostly late in pregnancy. The disease can progress rapidly, at times without warning, to a life-threatening disease. Expedient delivery initiates the resolution of pre-eclampsia but is a major cause of fetal and maternal morbidity and mortality.

Roberts et al in their classic article gathered the evidence to invoke activation of maternal endothelium as an underlying process. Generalized maternal endothelial cell dysfunction allowed most, if not all, clinical aspects to be potentially explained by a single, unifying process: hypertension through disturbed endothelial control of vascular tone, fluid retention by increased endothelial permeability, and clotting dysfunction resulting from abnormal endothelial expression of procoagulant. Eclampsia can be ascribed to focal cerebral ischemia resulting from vasoconstriction, consistent with the evidence of changes detected by new cerebral imaging techniques. The liver dysfunction intrinsic to the HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome could also be attributed to the effects of acute underperfusion.

Endothelial cells can be activated in several different ways that are potentially relevant to the origins of pre-eclampsia, and several candidate factors have emerged, including free fatty acids, lipoproteins, oxidized lipoproteins or lipid peroxides, tumor necrosis factor alpha (TNF-a), fibron ctin degradation products, and deported syncytiotrophoblastic microvillous fragments. The source of the factors that lead to endothelial cell dysfunction has not been determined with certainty, but the evidence points to the placenta.

In addition to endothelial dysfunction there is substantial published evidence that there is systemic activation of the maternal inflammatory cell responses in pre-eclampsia. Both granulocytes and monocytes are activated. There is increased release of the proinflammatory cytokines TNF-a and its 2 soluble receptors, interleukin 6 (IL-6) and soluble phospholipase A2 (an important mediator of inflammatory reactions) into the circulation. It is well known that the clotting system is abnormally activated, and complement systems are similarly affected. Postmortem observations indicate that in some circumstances the lethal pathologic condition resembles that of the Shwartzmann reaction, a particular form of inflammatory response to endotoxin that has been characterized in experimental animals. Since the above mentioned characteristics of pre-eclampsia resemble that of septic shock, we identified that also NMPF (IR) factor(s) are involved in pre-eclampsia that can worsen septic shock or sepsis. We addressed this by using a high dose LPS animal model for septic shock. Since in the urine of pre-eclamptic patients high levels of nicked hCG b-subunits are present, we also tested these nicked subunits to find out whether they worsen septic shock and so behave like MIF, which is an important mediator of lethal endotoxemia and staphylococcal toxic shock.

Material and Methods

[0065] NMPF purification: To analyse the NMPF from commercial hCG preparations, we used a Shimadzu HPLC system equipped with Alltech macrosphere size exclusion (GPC) column of 60Å, 100Å or 300Å (250 x 4.6 mm and 300 x 7.5 mm). The separation ranges of the columns were 28,000 - 250, 2500 - 350,00 and 1,200,000 - 7,500 Dalton, respectively. External molecular weight standards were employed to calibrate the column elution positions. The markers used were: aprotinin (6,500 Da), cytochrome C (12,400), carbonic anhydrase (29,000), albumin (66,000) and blue dextran (2,000,000).

To analyse NMPF, three different hCG preparations were used: NMPF-PG (Pregnyl; Organon; OSS, The Netherlands), NMPF-A (APL; Weyth Ayerst; Philadelphia, USA) and NMPF-PR (Profasi; Serono, Rome, Italy). As running buffer 50mM ammonium bicarbonate buffer containing ethanol (5%, vol/vol) was used. Sample load volume was 10-50 ml for the 250 x 4.6 mm column and 50-200 ml for the 300 x 7.5 mm column. The flow rate for the 250 x 4.6 mm and 300 x 7.5 mm columns were 0.5 ml/min for 45 min. and 1-2 ml/min for 45 min, respectively.

First trimester pregnancy urine (2 litres) was collected in a bottle from a healthy volunteer and was refrig rated until delivered at the laboratory within 2 days. Upon deliv ry, 1 gram per litr of sodium azide was added and the pH was adjust d to 7.2-7.4 with sodium hydroxid and allowed to sediment for 1 hour (h) at room temperature (RT). Approximately, 75% of the supernatant was decanted and the remainder close to the precipitate was centrifuged (10 min at 25,000 rpm at 40°C) to remove sediment and added to the rest of the supernatants. The supernatants were filtered through 0.45 mm in a Minitan (Millipore) transversal filtration set-up. Subsequently, the filtrate (2 litre) was concentrated in an Amicon ultrafiltration set-up equipped with an YM Diopore membrane with a 10 kDa cut-off. The final volume (250 ml) was dialysed against 2 changes of 10 litres of Milli Q water. Next the sample was further concentrated by 10 kDa cut-off in an Amicon ultrafiltration system to a final volume of 3 ml.

[0066] Mice used in sepsis or septic shock experiments: Female BALB/c mice of 8-12 weeks of age were used for all experiments. The animals were bred in our facility under specific pathogen-free conditions according to the protocols described in the Report of European Laboratory Animal Science Associations (FELASA) Working group on Animal Health (Laboratory Animals 28: 1-24, 1994).

[0067] Injection protocols: For the endotoxin model, BALB/c mice were injected i.p. with 150-300 µg LPS (E. coli 026:B6; Difco Lab., Detroit, MI, USA). Control groups were treated with PBS i.p. only. To test the effect of NMPF, we treated BALB/c with an optimized dose of 700 IU of different hCG preparations, thereof derived fractions (10-50 mg) or from first trimester pregnancy urine (NMPF-U) for 3 days and then injected with LPS i.p..

In order to determine whether NMPF inhibited shock even after the shock induction, we also treated BALB/c mice with NMPF i.p. after 3, 12, 24 and 36 h of injection with LPS. At different time points semi-quantitative sickness scores and survival rates were noted.

[0068] Semi-quantitative sickness measurements: Mice were scored for sickness severity using the following measurement scheme:

1 Percolated fur, but no detectable behaviour differences compared to normal mice.
2 Percolated fur, huddle reflex, responds to stimuli (such as tap on cage), just as active during handling as healthy mouse.
3 Slower response to tap on cage, passive or docile when handled, but still curious when alone in a new setting.
4 Lack of curiosity, little or no response to stimuli, quite immobile.
5 Laboured breathing, inability or slow to self-right after being rolled onto back (moribund, sacrificed).

b-hCG peptide and anti-MIF treatment:

[0069] Most urinary metabolites of hCG are a nicked form of b-hCG. These forms of b-hCG hav peptide bond cleavages within the b-subunit. b48 (VLPALPQVVC) is one such peptide which has been shown to be associated with a natural urinary metabolite of hCG. To test the effect of this peptide on septic shock, we injected BALB/c mice with LPS and treated them 2 h later i.p. with b48-peptide (100 mg). In order to see whether possible breakdown products also have effect on septic shock, we incubated b48-peptide at 37°C for three h before testing the peptide in the septic shock model in BALB/c mice.

Previously (WO 99-59617), we have shown that NMPF (IR) has also anti-diabetic effect. So in order to test whether b48 peptide has anti-diabetic effect, we performed transfer experiments. Total spleen cells were recovered from diabetic NOD mice and stimulated in vitro in RPMI+ supplemented with 10% FBS with coated anti-CD3 (145-2c11; 25 mg/ml) and IL-2 (50 U/ml) along with 300 IU/ml NMPF (Pregnyl) or b48 peptide (20 mg/ml). Culture flasks were then incubated at 37°C in 5% of CO_2 in air for 48 h. After 48 h cells were twice washed with PBS and 20 x 106 cells were i.p. transferred into an 8-wk-old NOD.scid mouse (n=4).

[0070] In vitro/ ex vivo LPS stimulated proliferation of splenocytes:

After 48 h of septic shock induction in BALB/c mice by high dose LPS injection, spleen cells (1 x 10^6 cells/ml) were recovered and restimulated *in vitro* with LPS (10 U/ml) in 96-well plates (round bottom). After 24 hours of culture, the LPS stimulated proliferation of splenocytes was measured via [^{3}H]TdR incorporation during the last 16 hours in culture. In other experiments splenocytes from non-treated BALB/c mice were isolated and in vitro stimulated (1 x 10^6 cells/ml) with LPS in the presence or absence of different sources of NMPF (37.5-600 IU/ml)(Pregnyl, Organon; APL, Wyeth Ayerst; Profasi, Serono), NMPF fractions (10-20 mg/ml), b-48 peptide or its breakdown products, anti-MIF or combinations of these products each at 10 mg/ml. After 24 hours of culture, the LPS stimulated proliferation of splenocytes was measured.

Results

[0071] NMPF purification: Samples of NMPF from different sources (Pregnyl, APL, Profasi, Pregnancy urine) were applied on the Macroshere GPC 300 Å column and eluted with ammonium bicarbonate. Three selected areas were fractionated, NMPF-1 which elutes apparently with molecular weight of >25 kDa, NMPF-2 which elutes apparently with molecular weight between the 25kDa-6kDa, and NMPF-3 which elutes apparently with molecular weight <6kDa (figure 1.). All these fractions were lyophilized and were t st d for anti-shock activity (shown elsewhere in this document). The lower molec-

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ular weight fraction (NMPF-3) which elutes after the column volume was furth r fractionated on the Macrosphere GPC 60 Å column (figure 2.). All fractions were lyophilized and were also tested for anti-shock activity.

[0072] NMPF treatment in LPS-induced septic shock: To determine the effect of high-dose LPS treatment in NMPF treated mice, BALB/c mice (n=6) were injected intraperitoneally with LPS (150 mg/kg) and survival was assessed daily for 5 days. PBS-treated BALB/c mice succumbed to shock from day 1 after high-dose LPS injection, with lower than 10% of mice alive on day 5 (figure 3.). In contrast, 100% of the mice treated with NMPF from source Pregnyl, or its fractions NMPF-1 or NMPF-3 obtained from GPC 300 Å column, were alive on day 5 ($P<0.001$) (figure 3.), while groups of mice treated with NMPF-2 from source Pregnyl or Dexamethasone (data not shown) demonstrated around 25% of survivors (figure 3). Not all commercial hCG preparations showed NMPF activity; for example NMPF from source Profasi showed only partial anti-shock activity (around 40% survival). In addition, variability in NMPF activity between different batches of the same source as well as variability of activity of same batch in time was observed. Treatment of BALB/c mice with APL before or after the shock induction, showed in a number of experiments acceleration of shock and early death.

[0073] In order to determine whether there are factor (s) present in hCG preparation that also accelerate shock and inhibit or counteract NMPF activity, we further fractionated NMPF-3 from a pretested active batch (containing anti-shock activity) and a non-active batch from source Pregnyl on GPC 60 Å column. Three selected areas were fractionated, NMPF-3.1 which elutes apparently with molecular weight of >2000 Da, NMPF-3.2 which elutes apparently with molecular weight between 2000-300 Da and NMPF-3.3 elutes apparently with molecular weight lower then 300 Da (figure 2.). All fractions were tested for anti-shock activity.

Results from these experiments revealed that anti-shock activity in a pretested active batch resided in a fraction NMPF-3.2, while NMPF-3.3 fraction from both (active and non-active) batches accelerated shock (figure 4.).

In order to determine whether NMPF-3.3 inhibits the anti-shock activity of NMPF-3.2, we added NMPF-3.3 into NMPF-3.2 in 10:1 ratio (100:10 mg) and injected the mixture i.p. in mice two hours after LPS injection (n=6). Data from these experiments showed that in all mice treated with NMPF-3.2 fraction alone, septic shock was inhibited and they had sickness scores lower than 2 (figure 4), while this anti-shock activity of NMPF-3.2 fraction was inhibited with NMPF-3.3. NMPF-3.3 treatment alone accelerated shock and the treated mice died even earlier than PBS treated mice (figure 4.). Same trend of results were obtained in experiments, in which active and non-active batches from Pregnyl were mixed and injected in BALB/c mice after septic shock induction (data not shown).

Ratio between NMPF-3.2 and NMPF-3.3: Next, we furth r purified NMPF-3.2 and NMPF-3.3 on GPC 60 Å column from active and non-active Pr gnyl batches, and from first trimester pregnancy urine and d termined the ratio. We found that first trimester pregnancy urine having anti-shock activity had around 1:2.2 ratio (NMPF-3.2 : NMPF-3.3) (figure 5.) and non-active batch of Pregnyl had 1:3.4 ratio (figure 6.), while the active batch of Pregnyl had around 1:1 ratio (Figure 7.).

Ex vivo LPS stimulated splenocytes proliferation: After 48 hours of LPS shock induction, splenocytes from PBS treated and NMPF treated mice(from mice treated with either active Pregnyl, thereof derived NMPF-3.2 or NMPF-3.3 fractions, or APL preparation) were isolated and restimulated with LPS. After 24 hours of culture, LPS stimulated proliferation of splenocytes was measured. Reduction in LPS induced proliferation was observed after culture of splenocytes from NMPF (active batch of Pregnyl) and thereof derived NMPF-3.2 (1600 vs 1350 cpm) fraction treated BALB/c mice as compared to PBS treated mice (3500 cpm), while treatment by NMPF(APL) or NMPF-3.3 increased the LPS stimulated proliferation (6000 vs 7200 cpm). Comparable results were obtained when splenocytes from untreated BALB/c mice were in vitro stimulated with LPS in the presence of above mentioned additions(data not shown).

[0074] In vitro treatment with NMPF from different sources, b-48 peptide, denaturated b-48 peptide and anti-MIF: The major characteristics of pre-eclampsia resemble that of septic shock. Therefore we hypothesized that there might be also NMPF (IR) factor(s)that are involved in pre-eclampsia and also worsen septic shock or sepsis. Above we have shown that NMPF-3.3 is one such fraction which accelerates septic shock and increases in vitro/ex vivo LPS induced splenocytes proliferation, which is correlated with increase in the disease severity. In the urine of pre-eclamptic patients high levels of nicked hCG b-subunits are present. Therefore we also tested whether these nicked subunits worse septic shock and so resemble NMPF-3.3 fraction. Furthermore, MIF is an important mediator of lethal endotoxemia and staphylococcal toxic shock, so we also compared the effects of b-48 peptide and NMPF on proliferation with anti-MIF and MIF.

These experiments revealed that anti-MIF has a trend to decrease LPS induced proliferation, similar as a pretested Pregnyl batch that shows anti-shock activity (NMPF-PG$^+$) (figure 8.). Moreover, anti-MIF and NMPF-PG$^+$ together work synergistically and decrease proliferation (figure 8.). NMPF from APL (NMPF-A), non-active Pregnyl batch (NMPF-PG$^-$; without anti-shock activity) and b-48 peptide (NMPF-K) increased the LPS induced proliferation as compared to LPS only (figure 8-12).On the other hand, NMPF-PG+ or denaturated b-48 peptide (NMPF-Kb) inhibited and decreased the LPS induced proliferation at least till the level of anti-MIF treatment alone (figure 8-12.). In vivo treatment of BALB/c mice with NMPF-PG$^-$, NMPF-K or NMPF-A after

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septic shock induction accelerated the disease severity (at t=48 hrs 0-25% survival rate) as compar d to PBS treated mic (at t=72 hrs 15% survival rate), while septic shock in BALB/c mice was completely inhibit d by NMPF-PG+ or NMPF-Kb.

In addition, our NOD spleen cells transfer experiments revealed that 22 days after transferring, NOD.scid mice receiving b48-peptide and PBS treated spleen cells were positive for diabetes and within a week they reached a blood glucose level above 30 mmol/1, while NOD.scid mice receiving NMPF (pregnyl) treated spleen cells remained normal (blood glucose <8 mmol/1). 6 weeks after transferring, the PBS and b48 reconstituted NOD.scid mice looked very uncomfortable, while NMPF mice group remained healthy. Mice from all groups were killed at this time.

[0075] There are many physiological conditions and immune pathologies where adaptive and innate immune systems are involved separately or in combination. For example, it has been shown that in pregnancy the maternal innate immune system is more stimulated, and it has been proposed that type II diabetes mellitus is due to chronic hyperactivation of the innate immune system. Another example is the involvement of the innate immune system in listeriosis. Dysregulation in the adaptive immune system may also lead to immune diseases like systemic or organ-specific autoimmunity, allergy, asthma etc, and the adaptive immune system can also play a role in the maintenance of pregnancy and in the prevention of "allograft" rejection and chronic inflammation, presumably including atherosclerosis and related diseases.

As shown in our previous (Immunoregulator; WO99-59617) NMPF (IR) is able to regulate the Th1/Th2 balance in vivo (BALB/c, NOD) and in vitro. In dominant Th1 phenotype models like NOD, NMPF (like NMPF-PG and its fractions) amongst others promote the IL-10 and TGF-beta production, which indicates the induction of regulatory cells like Th3 and Tr1 by NMPF. These regulatory cells may play role in the beneficial effects of NMPF in immune and inflammatory diseases and immune tolerance. While NMPF and several of its fractions are able to inhibit the production of IFN-gamma in vitro and in vivo, this was not observed for NMPF-3 (IR-P3) and recombinant hCG (rhCG). NMPF-3 (IR-P3) and rhCG separately show no to moderate inhibition of the IFN-gamma production, but the combination of NMPF-3 and rhCG gives a strong inhibition of the IFN-gamma production. This implies the need of NMPF-3 for rhCG for at least its IFN-gamma inhibition capacity in these models, while NPMPF-1 and NMPF-2 alone are capable to inhibit IFN-gamma production. This holds also for the anti-CD3 stimulated spleen cells obtained from in vivo treated NOD mice and for the polarization of T-helper cells to the Th2 phenotype. In our previous work we have also shown that NMPF (IR) has the potential to inhibit acute inflammatory responses, like in sepsis or septic shock. So, chronic as well as acut im-

mune responses are modulated by NMPF.

By way of example and not wishing to bound to theory, in pregnancy a fetus has to survive potential maternal immune rejection, which is in part achieved through d - viation of the maternal immune system towards Th2-type immune responses. But in this way maternal immune suppression carries the attendant risk of infection, as is observed in transplant patients receiving corticosteroids or other immunosuppressive therapy. NMPF (IR) factor(s) obtainable at least from pregnancy urine and thereof derived hCG preparations have the potential to modulate immune responses in such a way that the maternal rejection of the fetus is suppressed and that the mother maintains or even increases her resistance to infection. These and related factors are also responsible for the inhibition of immune diseases, particularly Th1-mediated immune diseases', during pregnancy.

By way of example and not wishing to bound to theory, pregnancy apparently demands incompatible immune adjustments. On the one hand, adaptive immune responses during pregnancy are modulated at different cellular levels towards immune tolerance state (such as Th2-type) and on the other hand the maternal innate immune system is modulated for resistance to infection. The evidence is that components of the maternal innate immune system are systemically activated. There are increased numbers of monocytes and granulocytes from the first trimester onwards. It has also been found that in normal pregnancy circulating monocytes and granulocytes in the maternal blood have an activated phenotype, in some ways comparable with changes observed in systemic sepsis. Others have shown increased monocyte phagocytosis and respiratory burst activity, and an increased expression of endotoxin receptor CD14 on monocytes as well as an increased response to endotoxin: monocytes from normal women produce more of the proinflammatory cytokines like in septic shock. Many studies have similarly found granulocyte activation in pregnancy as well as changes in plasma levels of soluble innate factors typical of an acute phase response. Not all components of the innate system are activated in the maternal circulation. Most notably, cytotoxic activity and IFN-gamma production by NK cells are suppressed.

By way of example and not wishing to bound to theory, we propose that one of the mechanisms of NMPF to modulate the immune response during pregnancy is the following: some NMPF factors during pregnancy can ensure that if T cells are activated, there is a bias to a Th2 response. This could be achieved by effecting different cell populations like macrophages, DC, T cells and their regulatory subsets. Other or similar NMPF factors could activate monocytes and hence other innate cells. So, the balance between different NMPF factors is crucial for a balanced regulation of different immune responses. We propose that in pre-eclampsia there is a misbalance between differ nt NMPF factors. Over-activation

of innate cells by NMPF factor(s) and/or a decrease in adaptive immune response (particularly Th1-type) inhibiting NMPF factor(s) could cause Th1/Th2 misbalance towards the Thl phenotype, in some ways comparable with changes observed in systemic sepsis. Our results showed that there are also NMPF factor(s) (NMPF-3.3) that can stimulate innate immunity and accelerate septic shock, while other NMPF factor(s) like NMPF-3.2 inhibit septic shock and the activity of NMPF-3.3. NMPF-3.2 factor(s) present in NMPF-3 fraction in combination with for example hCG modulate the adaptive immune response towards Th2-type (WO99-59617; inhibition of IFN-gamma by NMPF-3 (IR-P3) in combination with hCG) and is essential for normal pregnancy and inhibition of Th1 autoimmune diseases, induction of tolerance etc.

Analysis of hCG preparation (Pregnyl) and pregnancy urine have shown that hCG preparation and pregnancy urine having anti-shock activity contain NMPF-3.2 and NMPF-3.3 fractions in about an 1:2 ratio or higher, while hCG preparations without anti-shock activity or that worse septic shock have an NMPF-3.2 and NMPF-3.3 ratio of 1:3 or lower. This also explains why not all commercial hCG preparations have anti-shock activity. Moreover, we showed that hCG preparation possessing a high ratio of NMPF-3.3:NMPF-3.2 and so having no anti-shock activity, mixed with an active hCG preparation could gain anti-shock activity. So, the ratio between different NMPF factors or fractions like NMPF-3.2 and NMPF-3.3 can be used as a diagnostic marker not only for the prediction of successful pregnancy, but also for different immunopathology such as pre-eclampsia, sepsis or septic shock etc. In addition, in abnormal pregnancy like pre-eclampsia, one can also use NMPF factor (s) or NMPF-fraction(s) (e.g. NMPF-3.2) as a treatment. Our experiments also showed that NMPF (NMPF-3.2) inhibited septic shock even 30 h after shock induction, this shows that NMPF not only inhibits early mediators of endotoxin lethality like TNF-alpha, IL-1b, MIF, but also inhibits late mediators such as recently characterized high mobility group-1 (HMG-1) protein (Science 285, 248-251). hCG is a member of the structural superfamily of cysteine knot growth factors like NGF, PDGF-B and TGF-beta and a members of the glycoprotein hormone family which also includes LH, FSH and TSH. They each consist of two noncovalently associated protein subunits, a common 15 kD alpha chain and a hormone specific 23 kD beta chain (Annu. Rev. Biochem. 50, 465-495). hCG is produced by placental trophoblasts of normal pregnancy, and in gestational trophoblastic disease. It is also produced in much smaller quantities by the pituitary (Endocrinology 137, 1402-1411) in both pre- and postmenopausal women and in men (Trends in Endocrinology and Metabolism 1, 418-421), in many non-gestational malignant tumors and other tissues. hCG possesses a complex structure as a family of isoforms with structural, immunological and biological differences. The chemical basis for this het rogeneity is not known with certainty but differences in the amino acid composition, carbohydrate residu s or both have been proposed. More recently it was also shown that oxidation of specific methionine residues may also be responsible. Different forms of hCG, alpha and beta-subunits, their nicked fragments, beta-core fragment and multiple isoforms of hCG have been reported in different tissues and body fluids (Journal of Endocrinology 161, 99-106; Endocrinology 129, 1541-1550; Obstet. Gynecol. 77, 53-59; Journal of Biochemistry 107, 858-862; Obstet. Gynecol. 80, 223-228; Endocrinology 133, 985-989; 129, 1551-1558; 130, 2052-2058; Journal of Endocrinology 135, 175-188; 139, 519-532; Molecular and Cellular Endocrinology 125, 93-131).

Since all commercial hCG preparations are derived from pregnancy urine and contain different breakdown products of hCG, we speculated whether these products have NMPF activity. The most known breakdown products of hCG are beta-core hCG, a peptide bond nick in the beta-subunit between residues 44-45, 46-47 and 47-48. b48 (NMPF-K) is found in approximately 10-20% of the molecules in pregnancy urine and is associated with a natural urinary metabolite of hCG. Our experiments showed that NMPF-K accelerates septic shock (like MIF) and LPS induced proliferation of splenocytes alone or in combination with a non-active hCG preparation. This effect is inhabitable with anti-MIF, active hCG preparation, NMPF-3.2 and denaturated b48 (NMPF-Kb) peptide. This shows that NMPF-K activity resembles with NMPF-3.3 and the NMPF-Kb activity resembles to NMPF-3.2. In addition, there are also other peptide bond cleavages in hCG and its subunits as well as heterogeneity of the beta-core fragment. For example b45 bond cleavage, mainly found in hCG preparation and in urine, possibly derive from the action of bacterial proteases. In addition, Medeiros et. al. showed that HPLC separation of beta-core in its reduced and S-carboxymethylated forms showed three peptides, but only two of them could be sequenced and was demonstrated to be the previously reported b6-40 and b55-92 peptides of bhCG, while the third peak did not give any clear sequence because of the low signal due to several unidentified amino acids. We showed that breakdown products of NMPF-K share activity with NMPF-3.2. This NMPF-K peptide lies between two beta-core fragments (b6-40 and b55-92) and partially derived from beta-core b55-92 fragment. It is possible that there are also other single and/or double cleavage products of beta-core fragments or of not yet identified beta-core peptides (like Medeiros et. al. showed beta-core faction with a unidentified amino acids) responsible for NMPF activity in hCG preparations and pregnancy urine. Breakdown products of b48-peptide with additional unidentified amino acids from beta-core and/or with additional glycosylation possess among oth r anti-diabetic and anti-chronic inflammatory activity.

Figure l gends

[0076] Figure 1. This figur shows macrosphere GPC 300 A chromatogram of NMPF (Pr gnyl) sample. Three selected areas were fractionated, NMPF-1 which elutes apparently with molecular weight of >25 kDa, NMPF-2 which elutes apparently with molecular weight between the 25kDa-6kDa, and NMPF-3 which elutes apparently with molecular weight < 6kDa.

[0077] Figure 2. This figure shows macrosphere GPC 60A chromatogram of NMP-3 fraction obtained from macrosphere GPC 300 A column. Three selected areas were fractioned, NMPF-3.1 which elutes apparently with molecular weight between 2000-300 Da and NMPF-3.8 lutes apparently with molecular weight lower than 300 Da (figure 2.). All fractions were tested for anti-shock activity.

[0078] Figure 3. This figure shows that PBS-treated BALB/c mice succumbed to shock from day 1 after high-dose LPS injection, with lower than 10% of mice alive on day 5. In contrast, 100% of the mice treated with NMPF from source Pregnyl, or its fractionsNMPF-1 or NMPF-3 obtained from GPC 300 A column, were alive on day 5 (P<0.001), while groups of mice treated with NMPF-2 from source Pregnyl or Dexamethalsone (data not shown) demonstrated around 25% of survivors. Not all commercial hCG preparations showed NMPF activity; for example NMPF from source Profasi showed only partial anti-shock activity (around 40% survival).

[0079] Figure 4. This figure shows anti-shock activity in a pretested active batch resided in a fraction NMPF-3 and thereof derived NMPF-3.2 fraction which inhibit shock even after 24 hrs. and 36 hrs. of shock induction. In addition in all mice treated with NMPF-3.2 fraction alone, septic shock was inhibited and they had sickness scores lower than 2, while this anti-shock activity of NMPF-3.2 fraction was inhibited with NMPF-3.3. NMPF-3.3 treatment alone accelerated shock and the treated mice died even earlier than PBS treated mice.

[0080] Figure 5. This figure shows macrosphere GPC 60A chromatogram of pooled NMPF-3.2 and NMPF-3.3 fractions from first trimester pregnancy urine (containing anti-shock activity). This figure shows that the ratio between fraction NMPF-3.2 and NMPF-3.3 is around 1:2.2 (see text).

[0081] Figure 6. This figure shows macrosphere GPC 60A chromatogram of pooled NMPF-3.2 and NMPF-3.3 fractions from non-active Pergnyl batch (containing no anti-shock activity). This figure shows that the ratio between fraction NMPF-3.2 and NMPF-3.3 is around 1:3.4 (see text).

[0082] Figure 7. This figure shows macrosphere GPC 60A chromatogram of pooled NMPF-3.2 and NMPF-3.3 fractions from active Pergnyl batch (containing anti-shock activity). This figure shows that the ratio between fraction NMPF-3.2 and NMPF 3.3 is around 1:1 (see text).

[0083] Figure 8. This figure shows LPS induced proliferation of splenocytes. Anti-MIF and NMPF (from active Pr gnyl batch, NMPF-PG*) are both abl to decr ase LPS stimulated proliferation as compare to LPS alone, and together they show synergistically inhibitory effect on LPS stimulated proliferation.

[0084] Figure 9. This figure shows NMPF-A (APL) accelerate LPS induced proliferation, while this proliferation is inhibited by anti-MIFand NMPF-G*.

[0085] Figure 10 This figure shows that low molecular weight fraction(NMPF-PG3) from active Pregnyl batch (NMPF-PG*) as well as complete NMPF-PG+ are able to inhibit NMPF-A accelerated LPS induced proliferation.

[0086] Figure 11. This figure shows that NMPF-PG- (non-active Pregnyl batch) and NMPF-A or in combination (synergistically) increase LPS induced proliferation, while NMPF-PG* inhibits this proliferation same as anti-MIF (see figure 8-9).

[0087] Figure 12. This figure shows that NMPF-K accelerates LPS induced proliferation same as NMPF-PG-, while in combination they synergistically increas proliferation and this increase in proliferation is inhibited with NMPF-Kb or NMPF-PG*. In addition, NMPF-Kb and NMPF-PG* synergistically decrease LPS induced proliferation.

[0088] Figure 13-15: These figures show dose dependent (300 and 600 IU/ml) inhibitory effect of NMPF-PG+ on LPS and PHA/IL-2 induced proliferation of PBMC isolated from septic shock patient. Same effect was observed in medium conditions alone.

Claims

1. An immunoregulator obtainable or derivable from beta-HCG capable of regulating Th1 and/or Th2 cell activity.

2. An immunoregulator according to claim 1 capable of modulating dendritic cell differentiation.

3. An immunoregulator according to claim 1 capable of protecting a mouse against a lipopolysaccharide induced septic shock.

4. An immunoregulator according to claim 1 to 3 obtainable from nicked beta-HCG.

5. An immunoregulator according to anyone of claims 1 to 4 which comprises a peptide having an amino acid sequence MTRVLQGVLPALPQVVC or functional fragment or functional analogue thereof.

6. An immunoregulator according to claim 5 wherein said functional fragment comprises an amino acid sequence LQGVLPALPQVVC or functional analogue thereof.

7. An immunoregulator according to 6 wherein said functional fragment comprises an amino acid sequenc VLPALPQVVC or LQGVLPALPQ or functional analogue thereof.

8. An immunoregulator according to claim 7 wherein said functional fragment comprises an amino acid sequence VLPALPQ or GVLPALPQ or GVLPALP or VLPALP or functional analogue thereof.

9. An immunoregulator according to claim 8 capable of protecting a mouse against a lipopolysaccharide induced septic shock.

10. Use of an immunoregulator according to anyone of claims 1-9 for the production of a pharmaceutical composition for the treatment of an immune-mediated-disorder.

11. Use according to claim 10 wherein said immune-mediated disorder comprises chronic inflammation, such as diabetes, multiple sclerosis or chronic transplant rejection.

12. Use according to claim 10 wherein said immune-mediated disorder comprises acute inflammation, such as septic or anaphylactic shock or acute or hyper acute transplant rejection.

13. Use according to claim 10 wherein said immune-mediated disorder comprises auto-immune disease, such as systemic lupus erythematosus or rheumatoid arthritis.

14. Use according to claim 10 wherein said immune-mediated disorder comprises allergy, such as asthma or parasitic disease.

15. Use according to claim 10 wherein said immune-mediated disorder comprises an overly strong immune response directed against an infectious agent, such as a virus or bacterium.

16. Use according to claim 10 wherein said immune-mediated disorder comprises pre-eclampsia or another pregnancy related immune-mediated disorder.

17. Use according to claim 11 to 16 wherein said treatment comprises regulating relative ratios and/or cytokine activity of lymphocyte subset-populations in a treated individual.

18. Use according to claim 17 wherein said subset populations comprise Th1 or Th2 cells.

19. Use according to anyone of claims 11 to 18 wherein said immunoregulator comprises a hCG preparation or a fraction derived thereof.

20. A pharmaceutical composition for treating an immune-mediated disorder comprising an immunoregulator according to anyone of claims 1 to 9.

21. A pharmaceutical composition for treating an immune-mediated disorder comprising an active component obtainable from a urinary metabolite of HCG capable of protecting a mouse against a lipopolysaccharide induced septic shock.

22. A method for treating an immune-mediated-disorder comprising subjecting an animal to treatment with at least one immunoregulator according to any one of claims 1 to 9.

23. A method according to claim 22 wherein said disorder comprises diabetes.

24. A method according to claim 22 wherein said disorder comprises sepsis.

25. A method according to any one of claims 23 or 24 further comprising regulating relative ratios and /or cytokine activity of lymphocyte subset-populations in said animal.

26. A method according to claim 25 wherein said subset-populations comprise Th1 or Th2 cells.

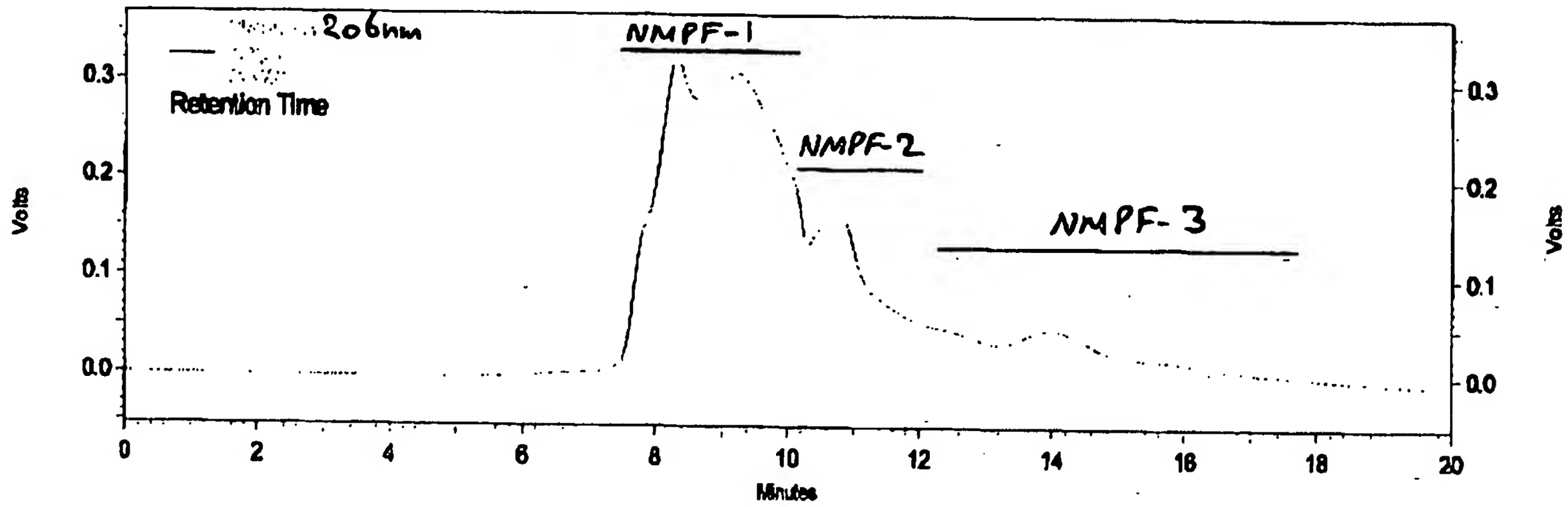
27. A method for diagnosing a pregnancy related immune-mediated disorder such as pre-eclampsia comprising determining in a sample, preferably a urine sample, the relative ratio of a relative long-chain peptide versus a relative short-chain peptide, said peptides derivable from breakdown of beta-HCG.

28. A method according to claim 27 comprising determining the relative ratio of a relative long-chain peptide versus a relative short-chain peptide derived from breakdown a peptide having an amino acid sequence MTRVLQGVLPALPQVVC.

29. A method according to claim 28 wherein said relative long-chain peptide comprises an amino acid sequence LQGVLPALPQ or GVLPALPQ or VLPALPQ or GVLPALP.

30. A method according to claim 27 or 28 wherein said relative short-chain peptide comprises MTRV or MTR or QVVC or VVC, or LQGV or LQG.

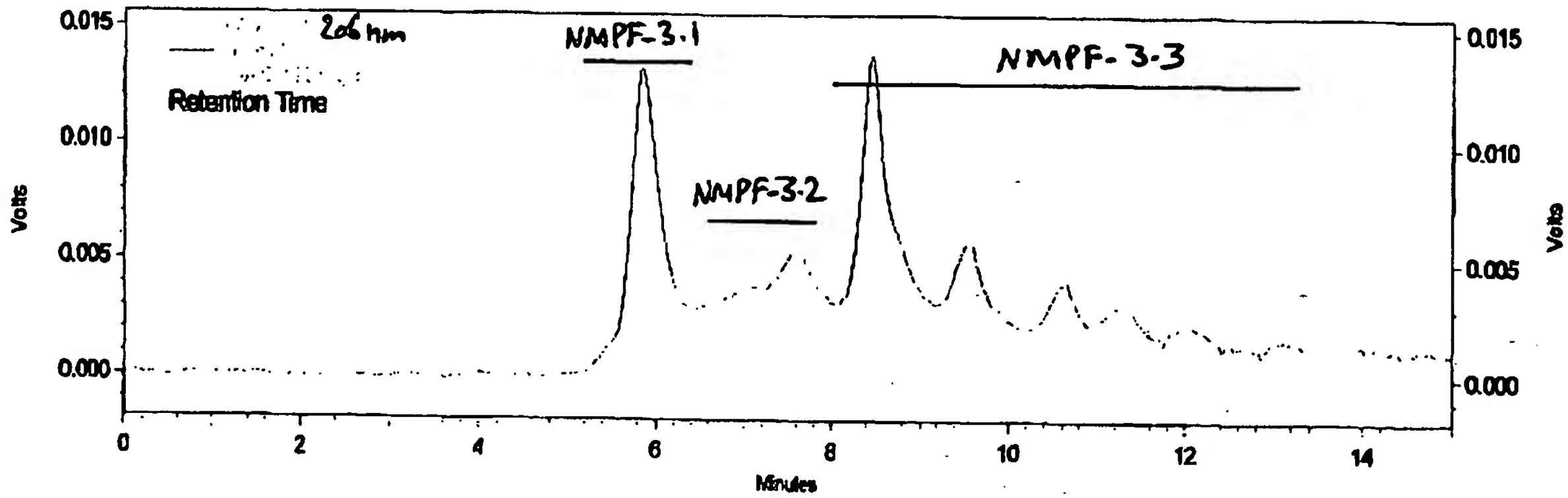
Figure 1.



This figure shows macrosphere GPC 300A chromatogram of NMPF (Pregnyl) sample. Three selected areas were fractionated, NMPF-1 which elutes apparently with molecular weight of >25 kDa, NMPF-2 which elutes apparently with molecular weight between the 25kDa-6kDa, and NMPF-3 which elutes apparently with molecular weight <6kDa

EP 1 138 692 A1

Figure 2.



This figure shows macrosphere GPC 60A chromatogram of NMP-3 fraction obtained from macrosphere GPC 300A column. Three selected areas were fractionated, NMPF-3.1 which elutes apparently with molecular weight of >2000 Da, NMPF-3.2 which elutes apparently with molecular weight between 2000-300 Da and NMPF-3.3 elutes apparently with molecular weight lower then 300 Da (figure 2.). All fractions were tested for anti-shock activity.

Figure 3

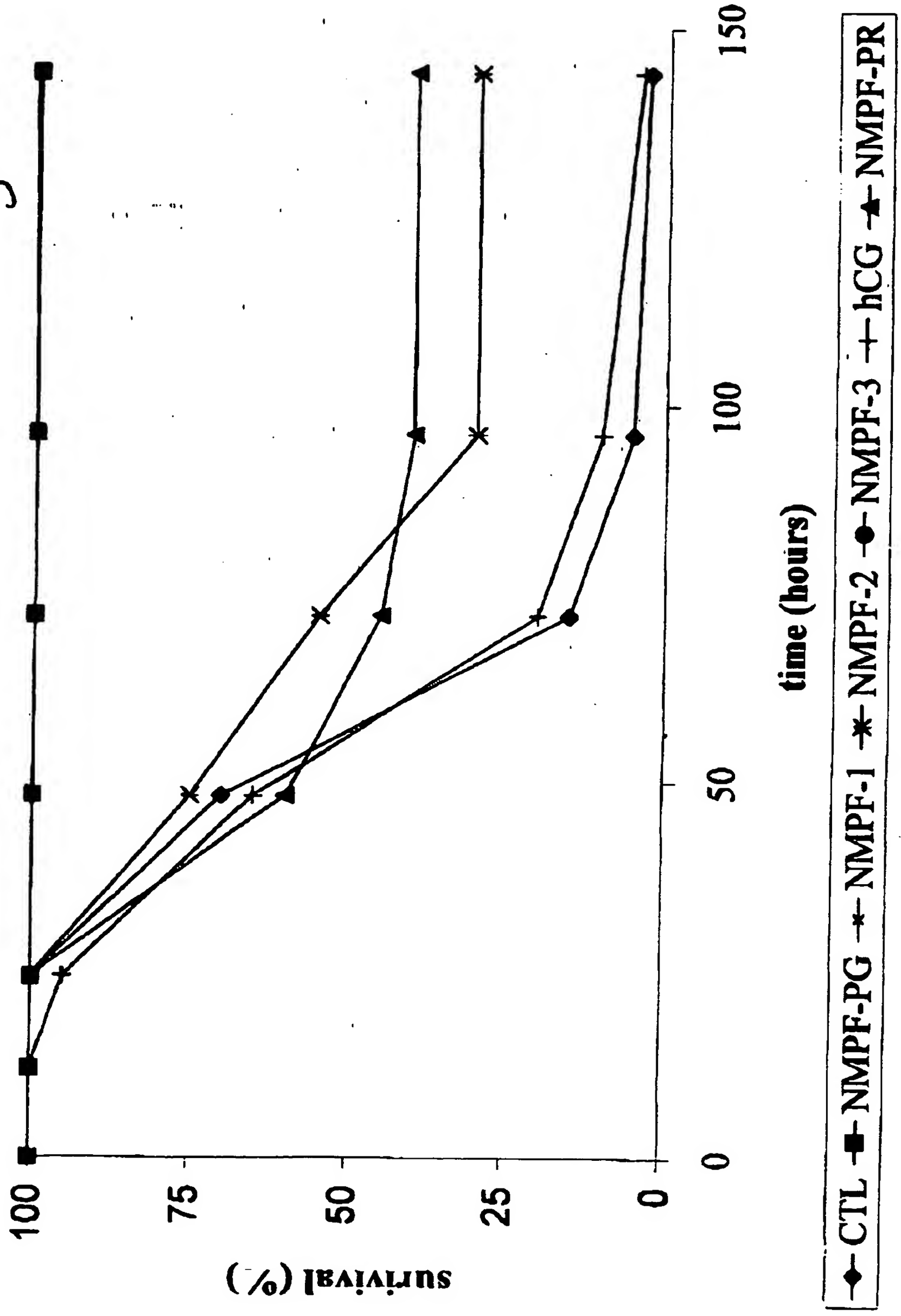
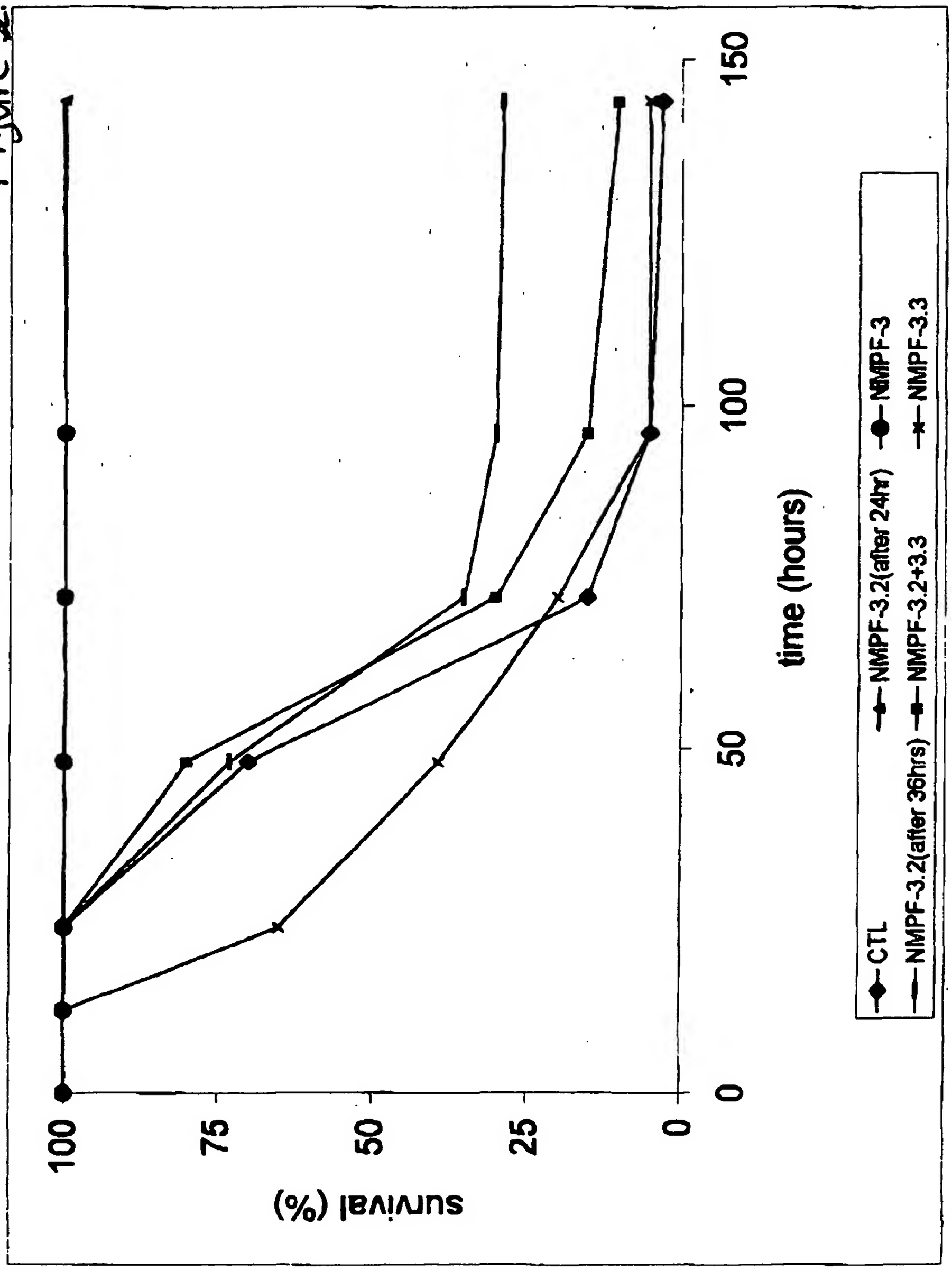


Figure 4



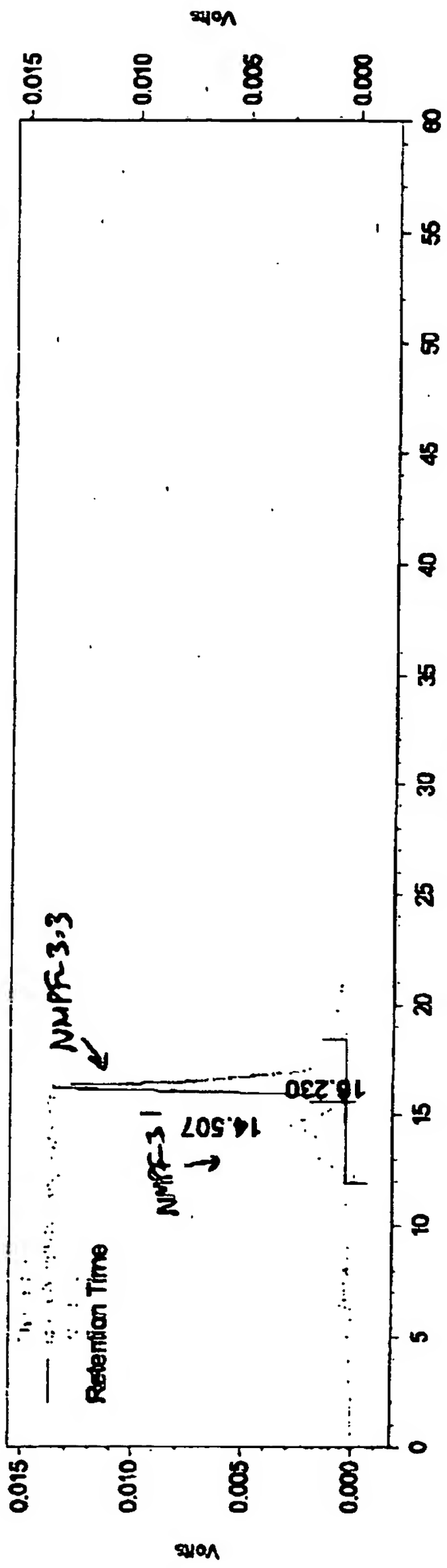


Figure 5.

This figure shows macrosphere GPC 60A chromatogram of pooled NMPF-3.2 and NMPF-3.3 fractions from first trimester pregnancy urine (containing anti-shock activity). This figure shows that the ratio between fraction NMPF-3.2 and NMPF-3.3 is around 1:2.2 (see text).

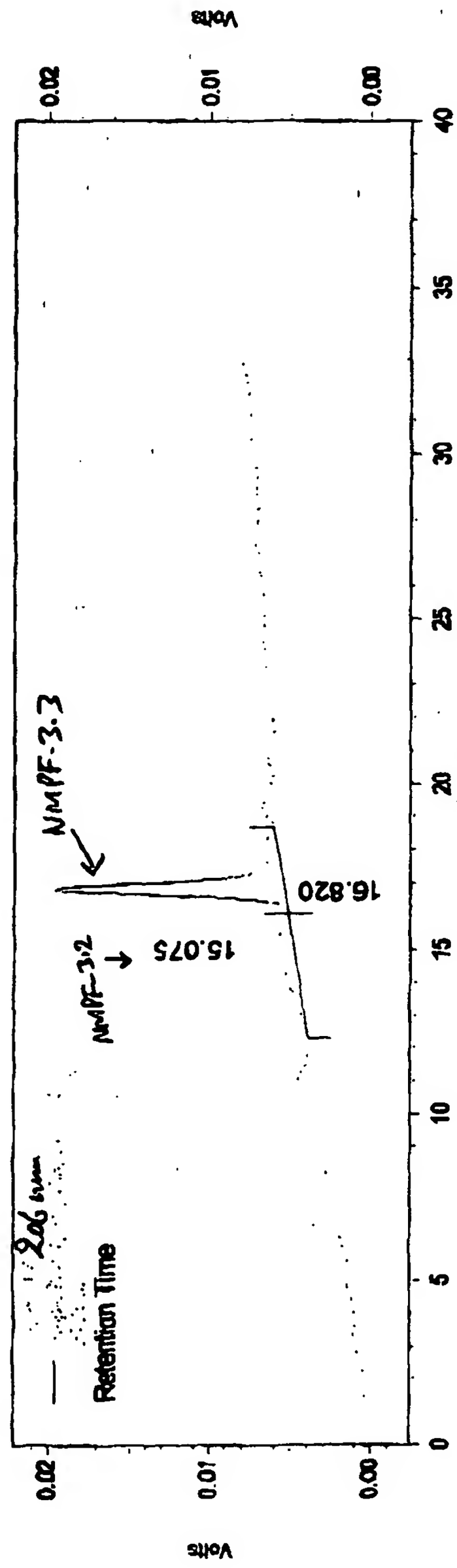


Figure 6.

This figure shows macrosphere GPC 60A chromatogram of pooled NMPF-3.2 and NMPF-3.3 fractions from non-active Pergnyl batch (containing no anti-shock activity). This figure shows that the ratio between fraction NMPF-3.2 and NMPF-3.3 is around 1:3.4 (see text).

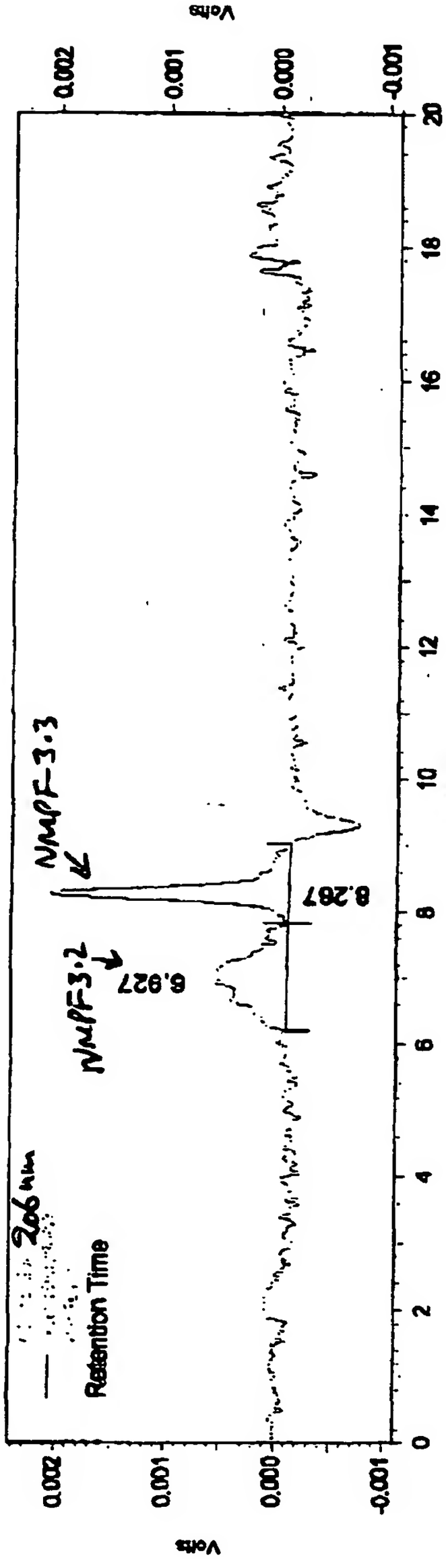


Figure 7.

This figure shows macrosphere GPC 60A chromatogram of pooled NMPF-3.2 and NMPF-3.3 fractions from active Pergnyl batch (containing anti-shock activity). This figure shows that the ratio between fraction NMPF-3.2 and NMPF-3.3 is around 1:1 (see text).

Figure 8

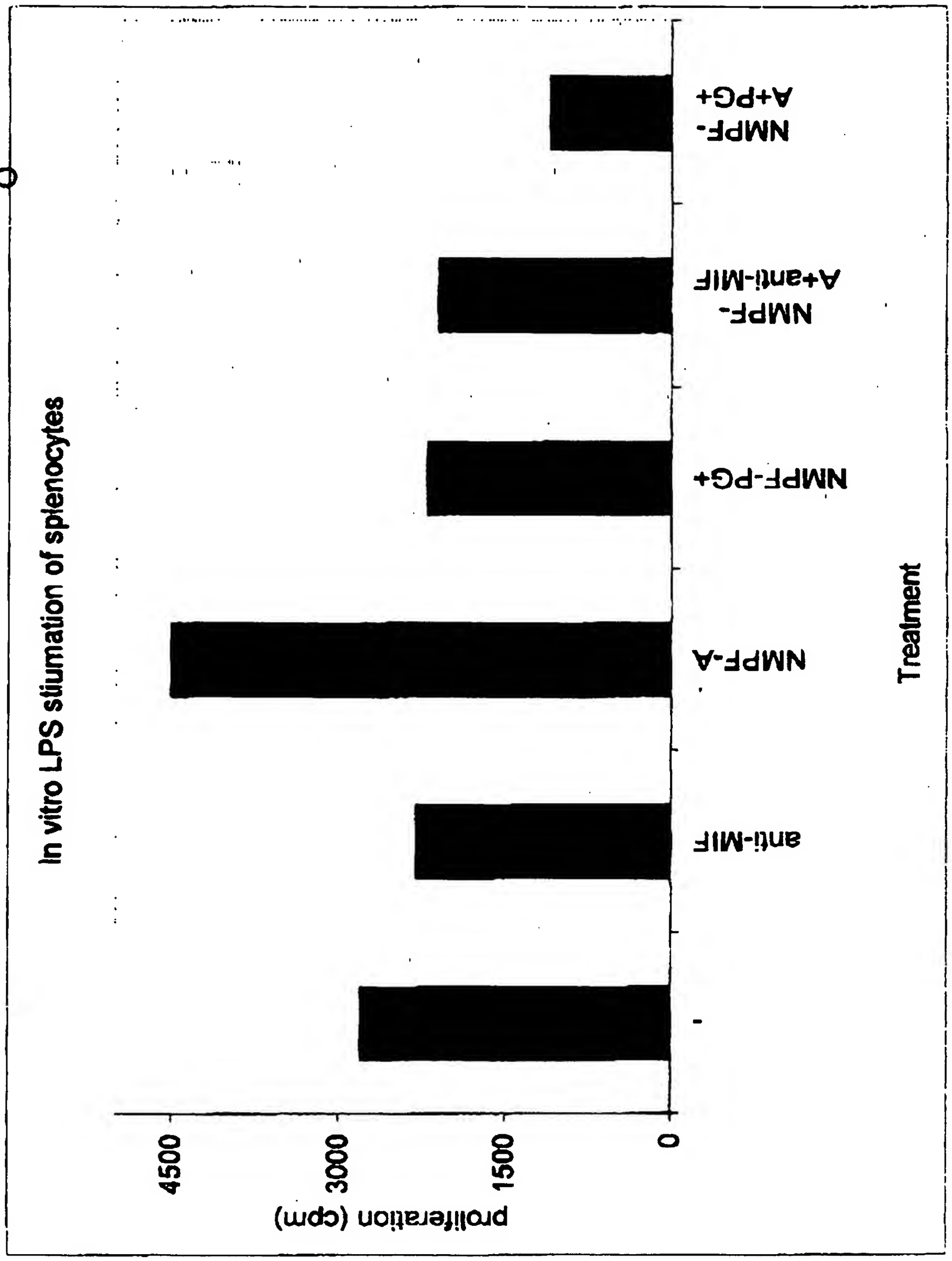

In vitro LPS stiumation of splenocytes
proliferation (cpm)
4500
3000
1500
0
Treatment
anti-MIF
NMPF-A
NMPF-PG+
-
NMPF-A+anti-MIF
NMPF-A+PG+

Figure 9.

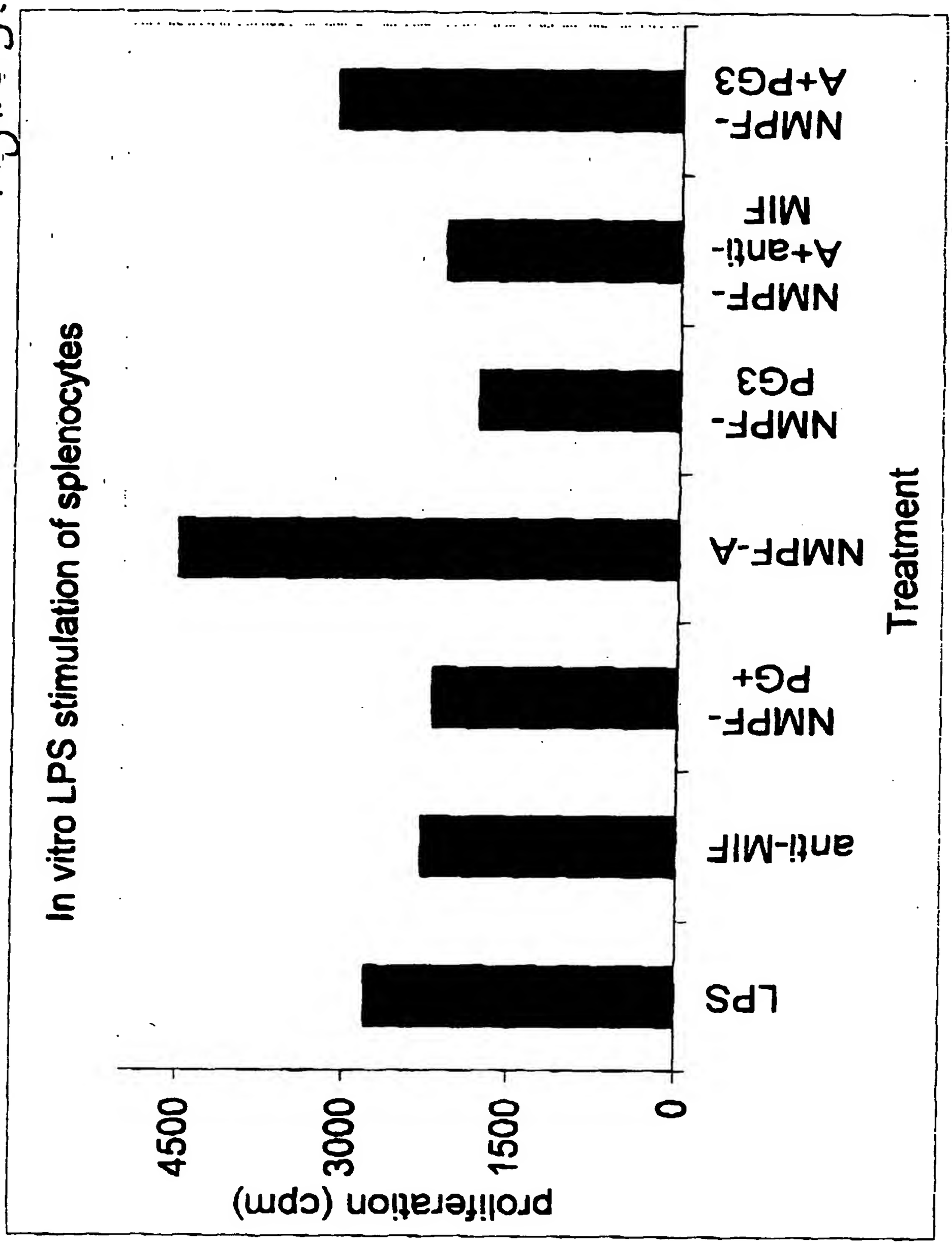


Figure 10.

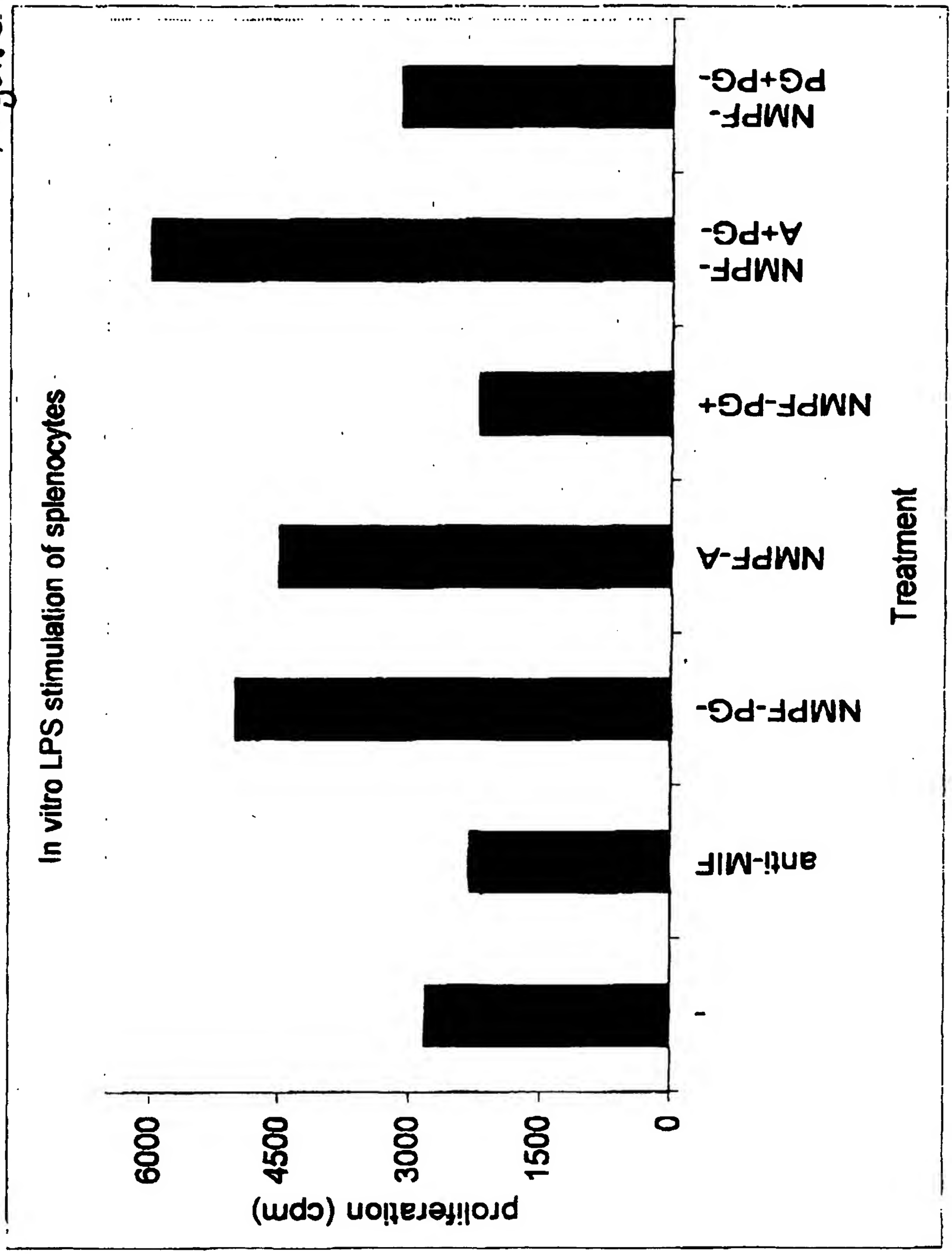


Figure 11

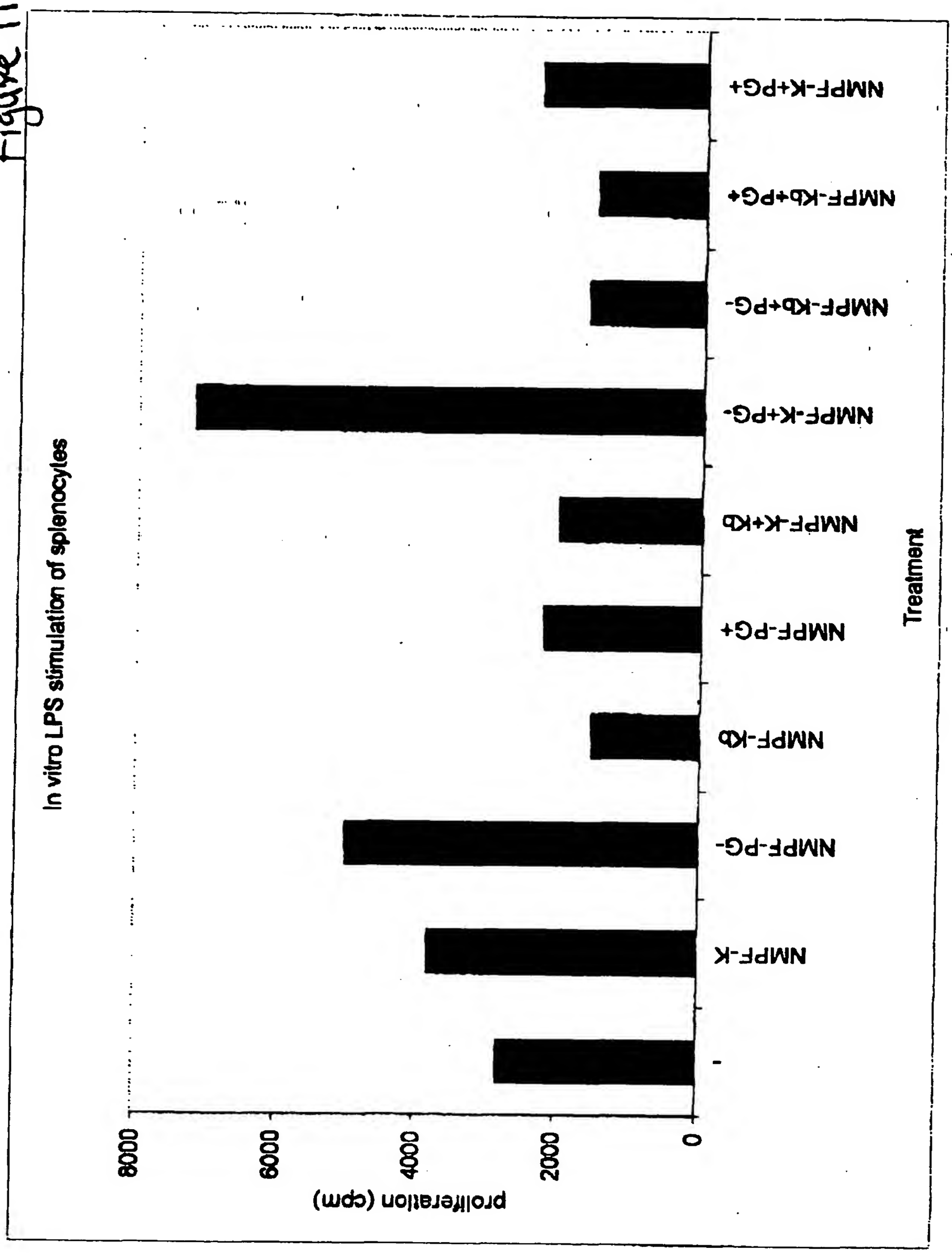


Figure 12

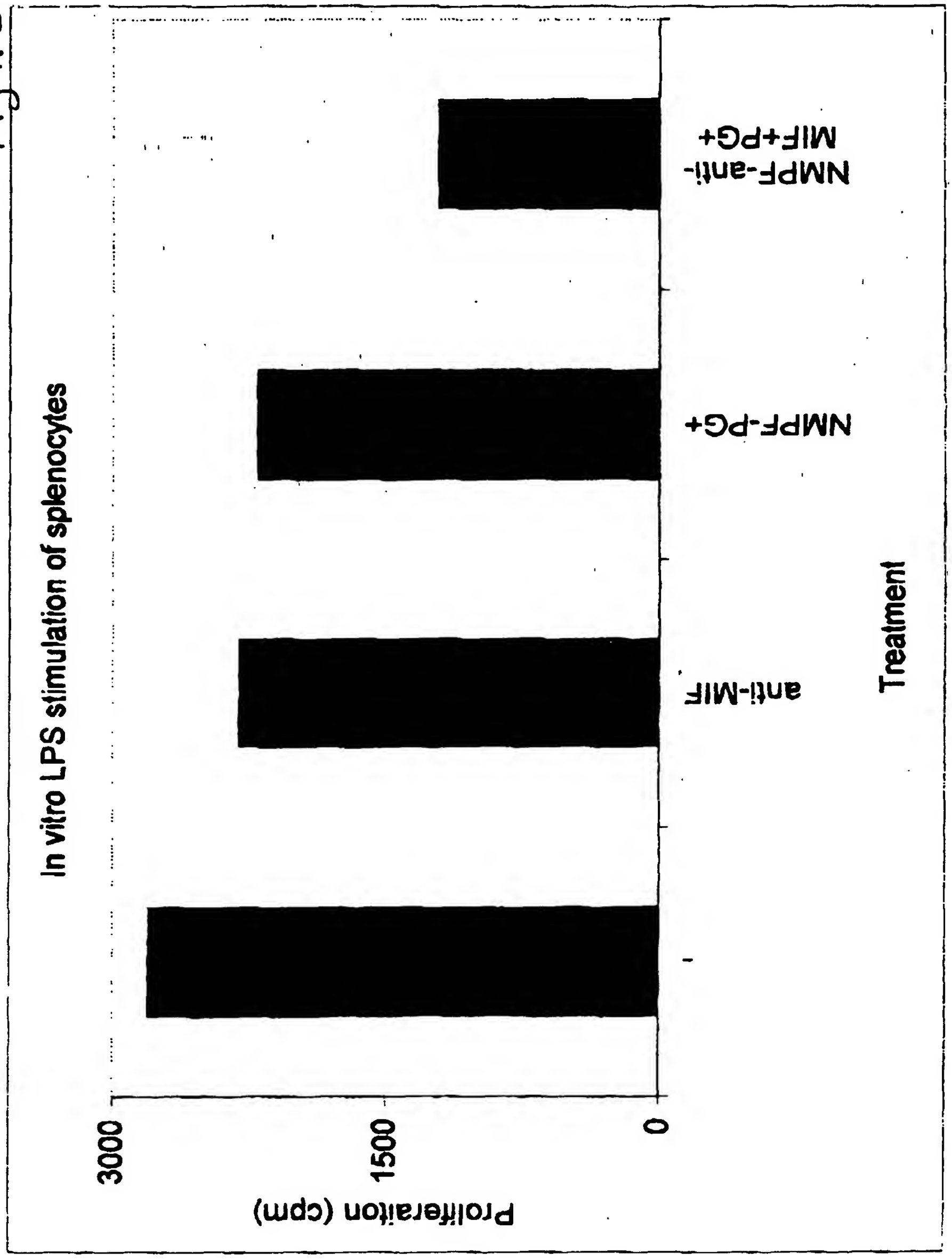


Figure 13

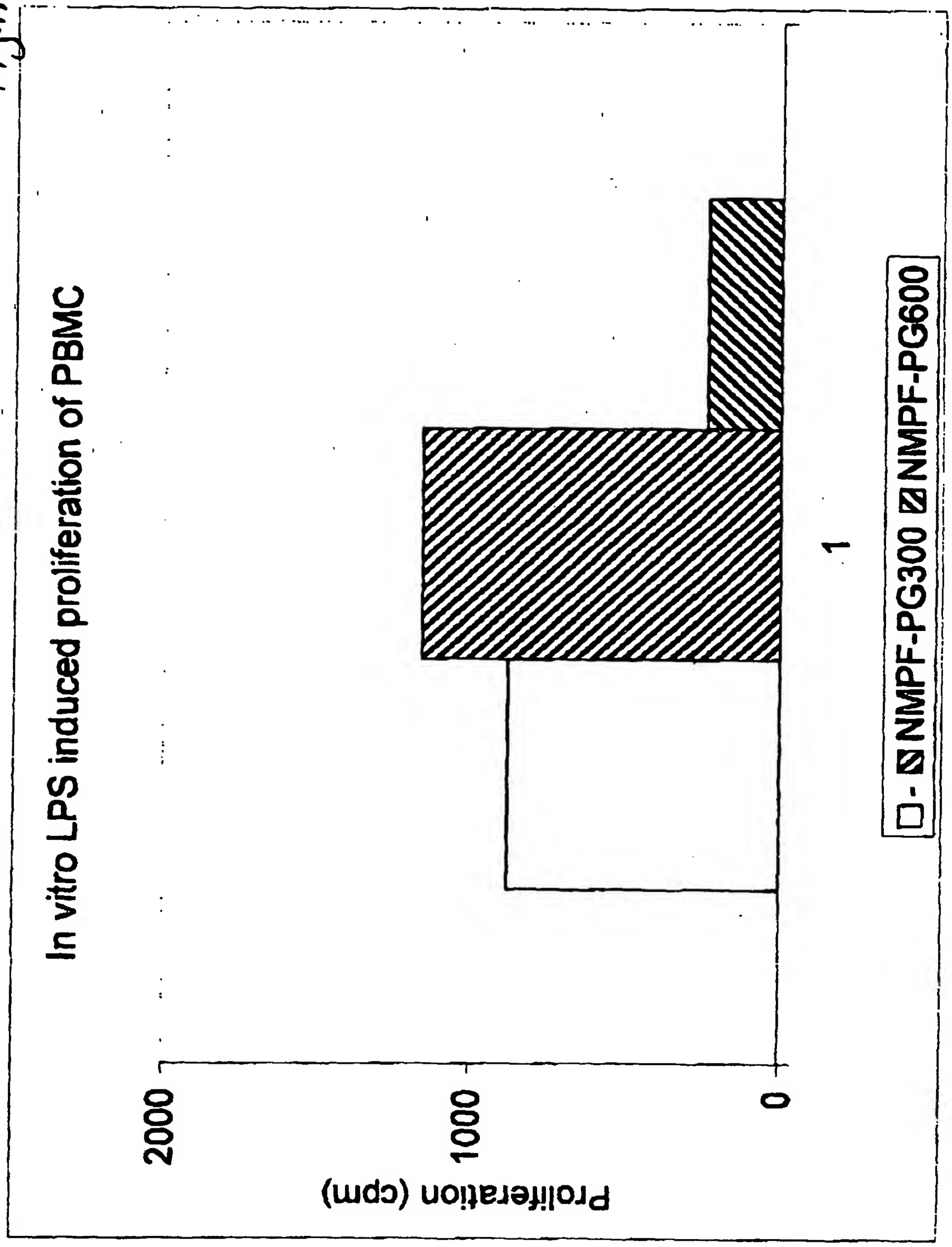


Figure 14

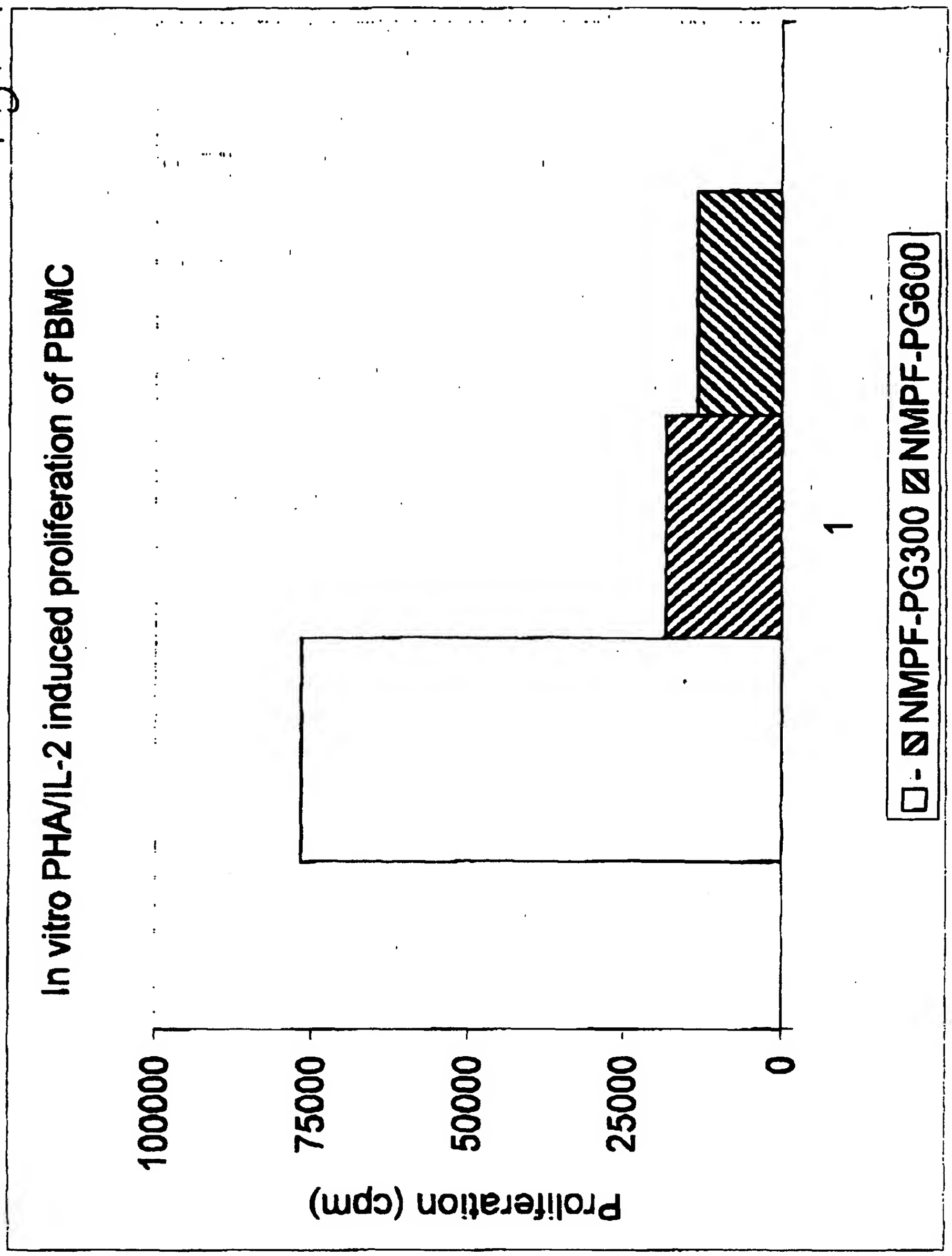
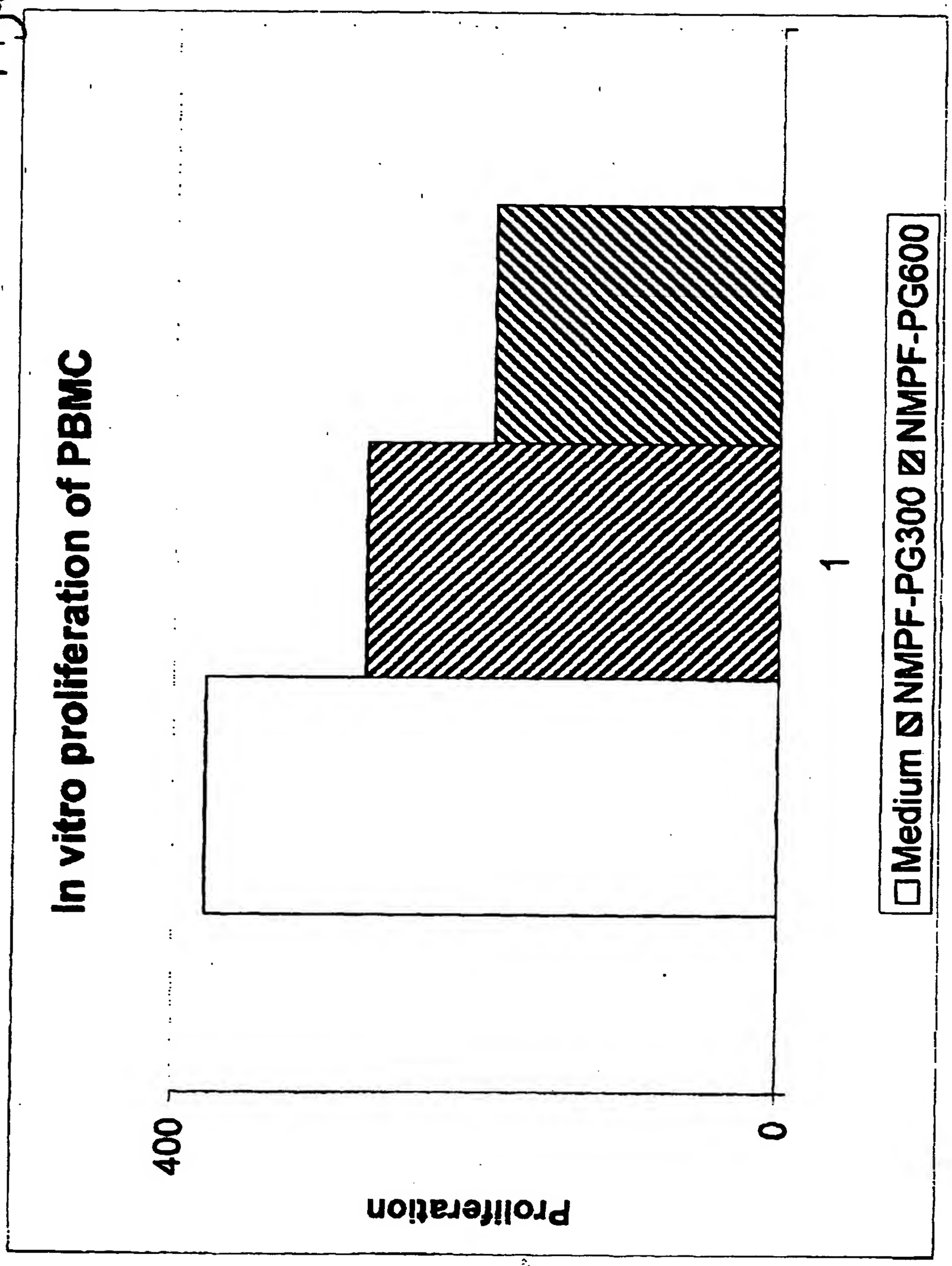


Figure 15



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Application Number EP 00 20 1139

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	WO 99 59617 A (SAVELKOUL HUBERTUS FRANCISCUS ;BENNER ROBBERT (NL); UNIV ERASMUS () 25 November 1999 (1999-11-25) * claims; examples *	1-30	C07K14/59 A61K38/24 G01N33/68 A61P37/02
X	WO 97 49721 A (BRYANT JOSEPH ;GALLO ROBERT C (US); LUNARDI ISKANDAR YANTO (US); U) 31 December 1997 (1997-12-31) * see Seq ID 19 * * claims; examples *	1-9,20, 21	

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

C07K
A61K
G01N

INCOMPLETE SEARCH

The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

Although claims 22-26 are directed to a method of treatment of the human/animal body (Article 52(4) EPC), the search has been carried out and based on the alleged effects of the compound/composition.

Place of search	Date of completion of the search	Examiner
THE HAGUE	7 September 2000	Fuhr, C

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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07-09-2000

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9959617 A	25-11-1999	EP 0958830 A	24-11-1999
		AU 4064399 A	06-12-1999
WO 9749721 A	31-12-1997	AU 3587797 A	14-01-1998

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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